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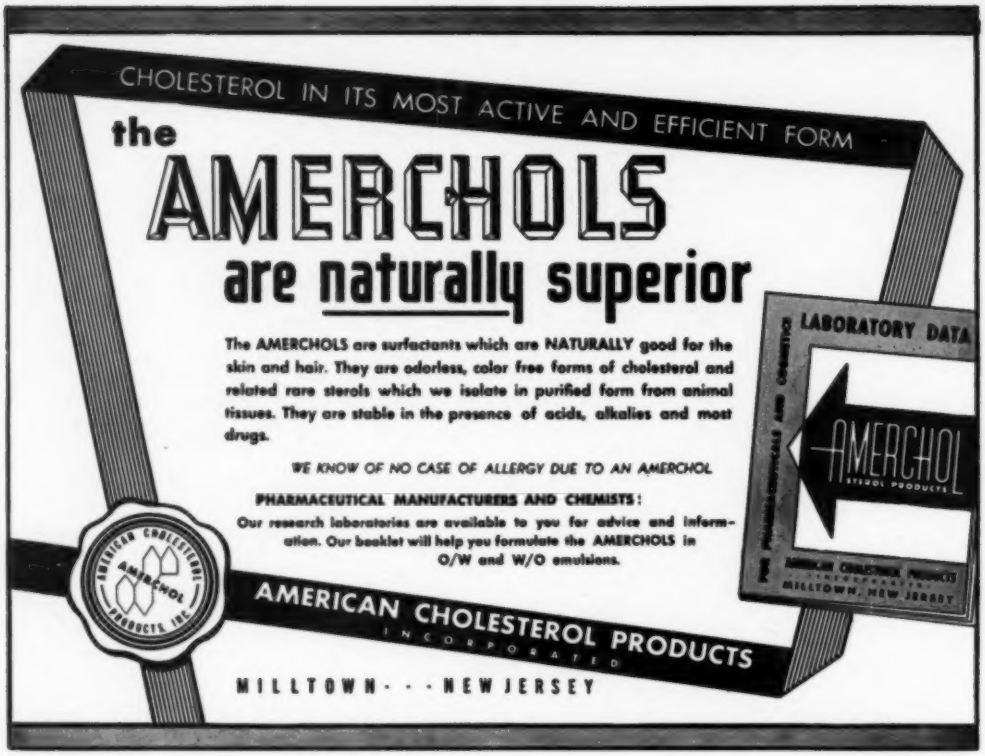
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Heavy Metal Determinations in Pharmaceuticals by Means of Amperometric Titrations*,†

By AARON D. COOPER‡ and MELVIN W. GREEN§

A procedure designed to eliminate visual differentiation of nonstandardized colors has been devised for the determination of microgram quantities of heavy metals. In this method, an excess of alcoholic *n*-dodecyl mercaptan solution is allowed to react with any heavy metals present. The excess reagent is then determined residually by titration with standard silver nitrate solution (carried out at −0.23 v. *vs.* S. C. E. in an ammoniacal medium to prevent chloride interference), using the rotating platinum electrode as the indicating electrode. A blank is run to compensate for possible oxidation of the mercaptan to the disulfide. Microgram quantities of Cu, Ag, Pb, Hg, and Cd were determined with precision and with an accuracy of within 0.5 p. p. m. over a range of 1.5 to 10 p. p. m. Additional applications were made on a number of inorganic and organic pharmaceuticals. Standardized conditions of assay for all pharmaceuticals were employed, the only variation being in the concentration of ammonium hydroxide used when chloride samples were assayed for their heavy metal content.

THERE is at the present time in the United States Pharmacopeia (1) and the National Formulary (2) a test for the determination of traces of heavy metals in various pharmaceutical substances. This test is designed to determine the approximate content of those metallic impurities in official substances which produce color in the presence of dissolved hydrogen sulfide due to the formation of the various colored sulfides. A visual comparison of the color produced is made between a known standard containing a specified amount of lead cation and the sample to be tested. The substance in question is required to possess a color which is not darker than that produced in the standard employed, and this limit is expressed as parts per million of lead present in one gram of sample.

The test employed at present is inadequate in a number of respects. It furnishes no exact value as to the amount of heavy metals present, since a comparison is merely made with a sample representing the maximum tolerance limit. The colors which are produced result from the formation of mixtures of different sulfides, each of which possesses its own characteristic color and contributes to the general color of the precipitate. Some substances, such as mercuric sulfide, tend to precipitate in a variety of colors, and the presence of any colloidal sulfur serves to introduce additional color to the suspension. Therefore, it is impossible to match with ease two entirely different colors, such as a brown

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† Abstracted from a thesis presented to the Graduate School of the University of Wisconsin by Aaron D. Cooper in partial fulfillment of the requirements for the degree of Master of Science.
‡ Fellow of the American Foundation for Pharmaceutical Education.
§ Associate Professor, School of Pharmacy, University of Wisconsin.

which may be produced by the unknown mixture and a black which is produced by the standard lead solution. A further point of interest arises where hydrochloric acid is employed in the medium, since it may be responsible for any turbidity which results from incomplete precipitation of the respective sulfides. A major difficulty lies in comparing the tubes containing the sample and the standard by eye and trying to decide which of the two possesses a darker color.

It is because of these and other inadequacies in the present method that an attempt has been made to devise a different procedure for the determination of traces of metals in pharmaceuticals.

A variety of methods has been employed in the quantitative determination of heavy metals present in microgram amounts, including colorimetry, emission spectroscopy, and various other methods. Among recent developments, amperometric titration with the rotating platinum electrode has provided a rapid and accurate method for determination, and it is upon this method that the work described in this paper is based.

To understand the principle behind amperometric titrations it is first necessary to examine the current-voltage relationship obtained in normal polarography. An outside depolarized reference electrode joined to a solution of constant concentration by means of a suitable salt bridge is employed, and a plot is made of the current flow through the circuit as a function of the change in the applied potential at a suitable indicating electrode. Up to a certain point (the decomposition potential), the current flow remains constant or increases slightly. Upon reaching this decomposition potential, the point at which the applied E. M. F. is sufficient to cause an electrode reaction, there is a sharp rise in the diffusion current due to the reaction taking place. The limiting value of this diffusion current is directly proportional to the concentration of electroreducible substance, provided all other factors are constant, and is caused by a virtually complete state of concentration polarization existing at the surface of the indicating electrode.

Unlike polarography, in carrying out amperometric titrations the applied potential on the system is maintained constant at a potential sufficiently beyond that necessary to produce an electrode reaction. The change in the current passing through the titration cell between the indicating electrode and an appropriate depolarized reference electrode is measured as a function of the volume of electroreducible substance added or removed. A rotating platinum electrode is employed as the indicating electrode, and is maintained at a potential sufficiently negative so as to permit reduction of electroreducible cations present in the solution. The current is measured by means of a microammeter.

The limiting value of the diffusion current at the rotating platinum electrode for a given concentration in solution is believed to be dependent upon two factors. The first of these is a diffusion of the electroactive ions in solution to the electrode surface, and the second is a transfer of electrons from the platinum electrode to the ions, causing an electrode reaction to take place.

ANALYTICAL PROCEDURE

Kolthoff and Harris (3) have shown previously that *n*-dodecyl mercaptan reacts quantitatively with a silver nitrate solution to yield a precipitate of silver mercaptide, and that this reaction could be followed amperometrically by measuring the diffusion current produced by the silver nitrate present in excess beyond the stoichiometric point. It was shown also that the reaction could be carried out in an acid or alkaline solution merely by varying the potential of the reference cell employed so as to produce an electrode reaction. Other workers (4–7) have made use of this reaction in the titration of microgram amounts of mercaptan sulfur compounds in various naturally occurring sources.

n-Dodecyl mercaptan, which has been selected as the reactive intermediate, possesses an acidic hydrogen on the functional group which is readily replaced by a number of inorganic cations to yield a quantitative precipitate of the mercaptide. It is upon this reaction that the present method has been developed for the determination of traces of heavy metals.

The procedure devised consists of allowing a measured excess of an alcoholic *n*-dodecyl mercaptan solution to react with a solution containing a standard amount of cation to be determined (Eq. 1):

$$MX + n\text{-}C_{12}H_{25}SH \rightarrow n\text{-}C_{12}H_{25}SM + HX$$

or

$$MX_2 + 2\ n\text{-}C_{12}H_{25}SH \rightarrow (n\text{-}C_{12}H_{25}S)_2M + 2HX \quad (1)$$

The excess *n*-dodecyl mercaptan is then determined by an amperometric titration, using a standard silver nitrate solution as the titrant in the presence of ammonium hydroxide, which is employed to prevent the precipitation of silver chloride and eliminate interference with the normal diffusion current. The reaction is shown in Eq. 2:

$$Ag(NH_3)_2X + n\text{-}C_{12}H_{25}SH \rightarrow n\text{-}C_{12}H_{25}SAg + NH_4X + NH_3 \quad (2)$$

The diffusion current obtained upon passing the stoichiometric point is recorded as a function of the volume of standard silver nitrate solution employed. The end point is determined graphically by plotting the diffusion current against the volume of standard reagent employed.

A blank is run at the same time on the *n*-dodecyl mercaptan solution to compensate for changes in strength through oxidation to the disulfide or evaporation of the alcohol used as solvent.

Reagents.—Standard silver nitrate solution (approximately 0.01 to 0.001 *N*) was employed as the titrant. This solution was prepared from primary standard silver nitrate dried for four hours at 80–90° and dissolved volumetrically in double-distilled water. This water was redistilled from an all-glass apparatus and was employed in the preparation of all solutions and titration media.

Alcoholic *n*-dodecyl mercaptan solution (approximately 0.0003 *N*) was prepared from *n*-dodecyl mercaptan (Eastman Kodak Co.). It is not necessary to know exact strength, as a blank is carried along with the sample.

Standard cation solutions prepared from cupric acetate monohydrate (molecular weight 199.67), lead nitrate (molecular weight 331.23), mercuric acetate (molecular weight 318.70), and silver nitrate (molecular weight 169.89) were made in a manner identical to the U. S. P. (1) method for the preparation of standard lead nitrate solution. The final dilution of each cation solution contains the equivalent of 1 μg./ml. and is rendered slightly acidic to prevent the precipitation of any neutral salts.

The following reagents were also used: hydroalcoholic (1:1) ammonium nitrate solution, 2.5 *M*; concentrated ammonium hydroxide solution; aqueous sodium bisulfite solution, 1 *M*; and absolute alcohol.

Instrumentation.—The apparatus employed (Fig. 1) was essentially the same as that described by Kolthoff and Harris (3) in their work, with certain modifications to adapt it to a microanalytical procedure.

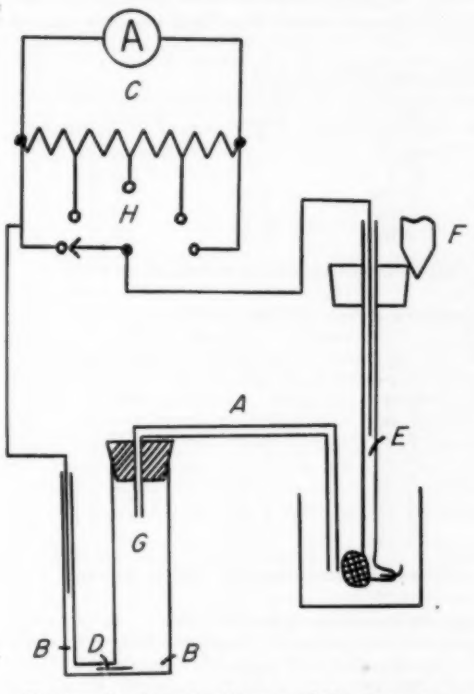

Fig. 1.—A schematic diagram of the apparatus.
A. Agar-gel bridge
B. Mercury resevoir contact
C. Microammeter
D. Platinum contact
E. Rotating platinum electrode
F. Sargent Çone Motor
G. Depolarized reference electrode
H. Variable shunt

The titration vessel employed was a 30-ml. beaker in which the rotating platinum electrode and agar-salt bridge were immersed. The agar-salt bridge was prepared from 3- or 4-mm. glass tubing of not more than 20 cm. in length and filled with a 3% agar and 30% ammonium nitrate salt-gel. Ammonium nitrate was chosen in preference to the conventional ammonium chloride to eliminate chloride leakage into the titration vessel.

The rotating platinum electrode was prepared from 6-mm. soft glass tubing 10–12 cm. long with the tip into which the platinum wire is sealed being drawn out to a thin cross section. The platinum wire employed was 0.5 mm. in diameter with the exposed length, about 5 mm., shaped into the form of an arc. The wire was sealed into the tip of the glass shaft so that a mercury contact was possible inside the tube. A glass wing tip was fused to the glass shaft on the side opposite the platinum wire to promote stirring in the solution. The glass shaft was mounted in the rotating holder of a Sargent cone motor with a variable speed adjustment, and electrical contact was made through a mercury pool reservoir in the shaft.

The agar-salt bridge was connected to a standard half cell of the Hildebrand type possessing a potential of −0.23 v. *vs.* S. C. E. (3).

The circuit between the rotating platinum electrode and the standard cell was connected through a mirror galvanometer (Rubicon) containing an Ayrton shunt in series. The galvanometer sensitivity employed may be varied depending upon the concentration of the silver nitrate solution used in the residual titration. It was found that the ideal sensitivity is 0.0195 to 0.0065 μa. per scale division.

Procedure.—Five milliliters of redistilled water and 8 ml. of absolute alcohol were mixed in a 30-ml. beaker and a volume of standard cation solution corresponding to the quantity for determination was added. Five milliliters of the mercaptan solution was added, followed by 2 ml. of the ammonium nitrate solution. Sodium bisulfite solution (0.3 ml.) and concentrated ammonium hydroxide (0.5 ml.) were added just prior to the start of the titration. The excess *n*-dodecyl mercaptan was then titrated with the standard silver nitrate solution. A blank was prepared and run in an identical manner. The difference in the number of milliliters of silver nitrate consumed by the sample and by the blank (Fig. 2) corresponds to the heavy metals content present, and for convenience is expressed in terms of lead.

Pharmaceuticals to be tested for their heavy metals content were first dissolved in the water or the alcohol, depending upon their solubility, and the other solutions were then added in the manner prescribed above. When the pharmaceutical being tested is precipitated from solution due to low solubility it is still possible to titrate its heavy metals content; however, it is advisable to employ a rotating platinum electrode without a wing-tip stirrer to prevent excessive agitation of the particles.

Discussion.—The first experiment made was the titration of the pure cations to determine their reactivity with *n*-dodecyl mercaptan and the accuracy of the residual method employed. Table I indi-

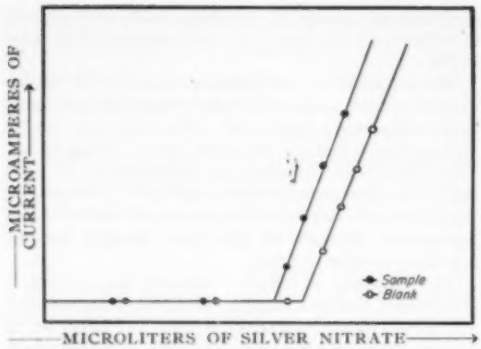

Fig. 2.—Titration curves of the blank and sample (microliter = 0.001 ml.).

cates the results obtained in the titration of lead ion in amounts down to 5 μg., and the percentage recovery falls within reasonable limits for an ultramicroanalysis. The silver nitrate solution employed as the titrant must be of such strength that it permits distinguishing between the titration curves representing the blank and the sample, as the difference between the volumes at the two end points represents the heavy metals content.

Tables II and III exhibit the recoveries obtained in the residual titration of cupric copper and mercuric mercury. The titrations involving copper have been carried out on samples as small as 1.5 μg. since the reaction takes place on a molar basis, and a smaller sample represents a larger molar quantity than is the case with lead or mercury since the atomic weight of copper is much smaller. As a consequence, it is much easier to distinguish between the titration curves of the blank and the sample as a larger volume of the *n*-dodecyl mercaptan solution is consumed by the cupric ions.

To distinguish between end points of the blank and the sample is one of the major problems encountered in this scheme of analysis. Solution strengths must be adjusted accordingly, burette readings must be accurately made, and current readings must exhibit a minimum fluctuation following each addition of reagent. An ultramicroburette calibrated in 0.001-ml. divisions is recommended, as the accuracy of the recovery is thus greatly enhanced (see Tables I, II, and III).

The residual titration is carried out at −0.23 v. *vs.* S. C. E. to prevent interference by chloride ion which may be present. At this potential, an ammoniacal solution of silver ions will undergo an electrode reaction, and the applied potential is not sufficiently negative to cause reduction of any dissolved oxygen present.

The ammonium hydroxide is added just prior to the start of the residual titration to prevent erroneous results. It has been shown by Strafford, *et al.* (8), that low results are obtained in the slow titration of an ammoniacal ethanol solution of mercaptan due to the formation of disulfide by oxidation.

Pharmaceuticals which were assayed for their heavy metals content were both inorganic and or-

TABLE I.—AMPEROMETRIC TITRATION OF LEAD ION

Burette Used[a]	No. Determinations	Lead Added, μg.	Av. Recovery, μg.	Range, μg.	Recovery, %
MB	1	50	49.2	..	98.4
MB	1	25	25.1	..	100.4
MB	2	10	9.78	0.25	97.8
MB	3	5	5.05	0.24	101.0
UMB	3	10	9.910	0.16	99.1

[a] MB: microburette calibrated in 0.01-ml. divisions. UMB: ultramicroburette calibrated in 0.001-ml. divisions.

TABLE II.—AMPEROMETRIC TITRATION OF CUPRIC COPPER ION

Burette Used[a]	No. Determinations	Copper Added, μg.	Av. Recovery, μg.	Range, μg.	Recovery, %
MB	1	50	51.4	..	102.8
MB	1	25	25.3	..	101.2
MB	3	10	9.87	0.07	98.7
MB	5	5	4.92	0.21	98.4
UMB	3	10	9.990	0.10	99.9
UMB	7	5	4.936	0.14	98.7
UMB	3	3	2.965	0.05	98.8
UMB	5	1.5	1.621	0.18	108.1

[a] MB: microburette calibrated in 0.01-ml. divisions. UMB: ultramicroburette calibrated in 0.001-ml. divisions.

TABLE III.—AMPEROMETRIC TITRATION OF MERCURIC MERCURY ION

Burette Used[a]	No. Determinations	Mercury Added, μg.	Av. Recovery, μg.	Range, μg.	Recovery, %
MB	2	25	24.7	0.00	98.8
MB	4	10	9.94	0.70	99.4
MB	6	5	5.02	0.14	100.4
UMB	2	10	9.991	0.00	99.9
UMB	2	5	5.045	0.00	100.9

[a] MB: microburette calibrated in 0.01-ml. divisions. UMB: ultramicroburette calibrated in 0.001-ml. divisions.

TABLE IV.—PHARMACEUTICALS ASSAYED FOR HEAVY METALS

Pharmaceutical Assayed	Official Heavy Metals Limit, P. P. M.	Heavy Metals Found (as Pb), P. P. M.	Heavy Metals Plus 10 P. P. M. Pb, P. P. M.[a]	Heavy Metals Initially Present, P. P. M.[b]
Aminopyrine	20	1.9	12.0	2.0
Benzocaine	10	13.3	23.4	13.4
Chloral hydrate	None	8.3	18.3	8.3
Lactose	5	5.6	16.0	6.0
Phenobarbital	None	Trace	..	..
Potassium nitrate	20	11.5	20.4	10.4
Magnesium sulfate	10	4.5	..	..
Procaine hydrochloride	None	1.0	..	..
Sodium chloride	5	4.7	14.7	4.7
Sodium phenobarbital	30	Fails	..	..
Sodium salicylate	20	3.5	13.2	3.2
Sodium sulfathiazole	20	Fails	..	..
Theobromine	20	Fails	..	..

[a] Standard addition method of assay.
[b] Figures in this column represent total heavy metals found minus amount added.

ganic in nature. In the method developed, a uniformity of conditions has been employed, with the sample being dissolved first in the aqueous or alcoholic portion of the medium, depending upon its solubility. The substances listed in Table IV were not specially purified samples, but were taken from bottles present in the dispensing laboratory which might account for several of the samples exceeding the official limit. One important limitation of the method is that substances which do not permit complete cathodic depolarization cannot be titrated under the conditions described and consequently fail to respond to the test.

CONCLUSIONS

As a result of experimental work it has been found that those cations which form insoluble sulfides will react with *n*-dodecyl mercaptan to give a precipitate of the metallic mercaptide. The solubility of this precipitate in an alkaline medium plays an important part in the determination of certain metallic cations. With substances such as silver, cupric copper, mercuric mercury, and lead, this metallic mercaptide is extremely insoluble and consequently the excess *n*-dodecyl mercaptan present may be titrated residually to determine that amount which has not reacted with the metallic ion species present.

Those substances which tend to form very insoluble hydroxides, such as ferric iron, cannot be determined by this procedure since the mercaptan consumed by the cation is liberated upon the addition of ammonium hydroxide and is subsequently titrated by the silver nitrate titrant. Ammonium hydroxide is necessary to prevent the precipitation of any silver chloride, which in turn interferes with the measurement of the diffusion current beyond the desired end point.

The reproducibility and accuracy of the results proved to be satisfactory. The accuracy fell within ±5% with a maximum absolute error generally falling within 0.2 μg. for the amount introduced. This absolute error is considerably smaller in cases where the equivalent weight of the substance in question is small, since it requires a larger volume of mercaptan to react with it; as a result, the difference between the volume of silver nitrate employed in the residual titration of the blank and the sample is larger. Since the reactions take place on a molar basis, substances possessing high equivalent weights require very small amounts of mercaptan solution; thus the difference in the standard silver nitrate solution consumed by the blank and the sample is very small and errors are magnified in the calculations.

SUMMARY

An amperometric titration procedure has been devised for the determination of traces of heavy metals in pharmaceutical chemicals.

Determinations have been made on microgram quantities of silver, cupric copper, mercuric mercury, and lead. Further applications were made on several inorganic and organic pharmaceuticals; in some cases, results have been compared with those obtained when samples from the same lot were tested by the standard addition method.

Standardized conditions of assay were employed for all pharmaceuticals tested by this procedure. Certain pharmaceuticals interfere with the test since they inhibit the attainment of complete cathodic depolarization under the conditions of the titration.

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The Selective Antibacterial Action of Phenylethyl Alcohol*

By B. D. LILLEY and J. H. BREWER

The inhibitory action of phenylethyl alcohol on the growth of several bacteria has been determined. The results reported indicate that phenylethyl alcohol in relatively low concentrations (1:400) exerts an effective inhibitory action on Gram-negative bacteria and may thus be used for differential inhibition.

IN A STUDY of several related organic compounds, phenylethyl alcohol was noted to exhibit a marked inhibitory effect on Gram-negative bacteria while inhibiting the Gram-positive organisms only slightly. In view of this differential inhibition, the compound seemed to warrant trial as an inhibitory agent in basal bacteriological culture media. It is the purpose of this paper to demonstrate the extent to which phenylethyl alcohol does inhibit many bacteria, thus aiding in the isolation of others, and to present the optimum concentration for several basal media.

METHODS AND MATERIALS

Phenylethyl alcohol[1] (benzylcarbinol, $C_6H_5CH_2CH_2OH$) occurs in the volatile oil of the rose and many other flowers. It is a colorless liquid, soluble in water; b. p. 219–222°. It may be synthetically prepared by reduction of ethyl phenylacetate with sodium in absolute alcohol.

Most of the organisms employed in this study were isolated from autopsies at the Johns Hopkins Hospital. *Micrococcus pyogenes* var. *aureus* 209P is an FDA strain; *E. coli* is a laboratory stock strain of Hynson, Westcott & Dunning. The basal media used were all dehydrated culture media obtained from commercial sources.

To find the optimum concentration for differential inhibition, varying concentrations of phenylethyl alcohol were prepared and tested in infusion broth with and without addition of serum. An infusion agar with several dilutions of phenylethyl alcohol was also inoculated by streaking the surface of poured 3% blood plates with twenty-four hour mixed Gram-positive and negative cultures. Colonial morphology of a number of streptococci and *D. pneumoniae* II was also observed on infusion agar containing 0.25% phenylethyl alcohol, and growth comparisons were made with 3% rabbit, human, and sheep defibrinated blood.

RESULTS

Table I shows the extent to which varying concentrations of phenylethyl alcohol inhibited a number of Gram-positive and negative organisms in infusion broth with and without enrichment. A compound dilution of 1:200 proved to be too concentrated for any growth, while 1:400 (0.25%) concentration approaches optimum conditions for isolation.

Data in Table II indicate the effectiveness of phenylethyl alcohol as an inhibitory agent in solid media. The appearance of both Gram-positive and negative colonies in concentrations of 0.2, 0.25, and 0.3% after twenty-four and forty-eight hours of growth is described. The size of the colonies, the hemolysis, and the color were changed and harder to recognize, although all types were easily seen and well isolated. *Proteus mirabilis* J2 appeared as very small, shiny, colorless, nonspreading colonies. With 0.25% concentration of phenylethyl alcohol, *Micrococcus pyogenes* var. *aureus* 209P was nearly the same size as the control, although the pigment appeared bleached. The zone produced by *Streptococcus* C-203 was smaller and the colony very tiny, but examination indicated beta hemolysis. The pneumococcus colony was very small; however, it showed alpha hemolysis and was easily transferred to broth. Although there are changes in the size of the colony and degree of hemolysis when grown on media containing an appropriate concentration of phenylethyl alcohol, organisms retain a recognizable morphology. In both solid and liquid media, a 0.3% preparation was toxic for most bacteria including the Gram-positives, whereas a 0.2% preparation did not sufficiently inhibit the Gram-negative spreaders or prevent others of the same group from hemolyzing the blood. As shown in Table II, the most effective concentration for studies was 0.25%.

* Received December 5, 1952, from the Department of Biological Research, Hynson, Westcott & Dunning, Inc., Baltimore, Md.
[1] Magnus, Mabee & Reynard, Inc., New York.

TABLE I.—GROWTH IN INFUSION BROTH MEDIUM CONTAINING VARIOUS AMOUNTS OF PHENYLETHYL ALCOHOL[a]

Phenylethyl Alcohol Dilution	1:200		1:400		1:800		1:1600		1:3200	
	Serum	Without Serum	Serum	Without Serum	Serum	Without Serum	Serum	Without Serum	Serum	Without Serum
Salmonella typhosa	−	−	+	−	+	−	++	++	++++	++++
Salmonella enteritidis, var. *danyx*	−	−	+	−	++	+++	+++	++++	++++	.....
Pseudomonas aeruginosa	−	−	+	−	++	+	+++	++++	++++	.....
Proteus mirabilis J2	−	−	+	+	+++	++	++++	++++	.....	.....
Aerobacter aerogenes	−	−	+	+	++	+++	++++	++++	.....	.....
Friedlander's bacillus	−	−	+	+	+++	+++	++++	++++	.....	.....
Escherichia coli	−	−	+	+	++	+++	+++	++++	++++	.....
Shigella	−	−	+	−	+++	+++	++++	++++	.....	.....
Micrococcus pyogenes, var. *aureus* 209P	+	+	+++	++	++++	++++	.....	.....	.....	.....
Streptococcus C-203	−	..	+	..	+++	.....	++++	.....	.....	.....

[a] Growth graded in turbidity: −, no growth, to ++++, maximum growth. Johns Hopkins cultures except F. D. A. strain 209P.

TABLE II.—GROWTH COMPARISON ON AGAR CONTAINING PHENYLETHYL ALCOHOL[a]

Phenylethyl Alcohol Concentration, % → Incubation at 37°, Hr. →	0.3		0.25		0.2		Control
	24	48	24	48	24	48	24
D. pneumoniae II and *P. mirabilis* J2	No growth	No growth	Well-isolated colonies of both; too small to see clearly	Pneumococcus larger, not sharp hemolysis, definite greening; few *Proteus;* no spreading	Predominance of *Proteus;* Pneumococci seen, still small; no spreading	Too dark	Overgrown
Streptococcus C-203 and *P. mirabilis* J2	Few C-203, very small	Clearly isolated C-203; no *Proteus*	Best plate; mostly C-203 isolated and large enough to see clearly	Too dark	Many well-isolated colonies of both large enough to fish to broth	Too dark	Overgrown
Streptococcus C-203 and *Pseudomonas aeruginosa* K1	Pinpoint *Pseudomonas aeruginosa;* C-203 small, dominant	Hemolyzed, cannot see C-203	Many, well isolated colonies	Completely hemolyzed	Well-isolated colonies	Completely hemoloyzed	Overgrown
D. pneumoniae II	No growth	No growth	Few colonies, small	Many colonies	Many colonies	Typical, many isolated colonies	Typical, many isolated colonies
Streptococcus C-203	Few colonies, small	Many, well-isolated colonies	Many colonies	Typical, many isolated colonies	Typical, many isolated colonies	Typical, many isolated colonies	Typical, many isolated colonies
P. mirabilis J2	No growth	No growth	Few colonies	Many isolated colonies	Many isolated colonies	Typical, many isolated colonies	Typically overgrown
Pseudomonas aeruginosa K1	Few colonies	Many colonies; hemolytic	Few colonies	Many colonies; hemolytic	Many colonies	Many colonies; no spreading; hemolytic	Typical, very hemolytic; few isolated

[a] Mixed broth cultures before streaking plates.

TABLE III.—APPEARANCE OF COLONIES ON BLOOD AGAR WITH AND WITHOUT 0.25% PHENYLETHYL ALCOHOL, USING THREE KINDS OF BLOOD[a]

Organisms Streaked	Infusion Agar Control Medium	Infusion Agar with Phenylethyl Alcohol
Diplococcus pneumoniae II	Rabbit—third best Human—largest colony Sheep—sharpest zone	Rabbit and human—same Sheep—sharpest zones (smaller than control colonies) easily seen Rabbit, human, sheep alike in size
Streptococcus pyogenes X 40	Rabbit and Human—same Sheep—largest, sharpest zones	Rabbit and human—same Sheep—sharpest zones (tiny colonies compared to control) Rabbit, human, sheep alike in size
Streptococcus LD3 group D, *Beta*-hemolytic	Rabbit—largest, sharpest zones Human—same colony size as rabbit Sheep—tiny colony, no zone	Rabbit—largest zone Human—next largest zone Sheep—smallest zone, but sharpest (colonies smaller but much larger zones than control) Rabbit, human, sheep alike in size
Streptococcus DZ-7874 group B, *Beta*-hemolytic	Rabbit and human—same Sheep—same size colony as rabbit and human, sharpest zones	Rabbit—colony size of sheep, zone smaller Human—smallest colony and zone Sheep—largest, sharpest zone
Streptococcus NC18 group B, *Beta*-hemolytic	Rabbit—same size zones as sheep Human—no zones Sheep—sharpest zones	Rabbit—large colony, small zone Human—large colony, small zone Sheep—smaller colony, large sharp zone
Streptococcus mitis	Rabbit, human, sheep—same	Rabbit, human, sheep—same, not much smaller than control

[a] Cultures obtained from Johns Hopkins autopsy laboratory.

Table III shows the wide variation which occurs in colony size and hemolysis when different kinds of blood are used with phenylethyl alcohol in an infusion agar medium. Phenylethyl alcohol in 0.25% concentration proved most satisfactory in a wide number of basal media such as infusion broth and agar, trypticase soy agar, anaerobic agar, and Sabouraud's dextrose agar. Anaerobes were easily isolated when contaminated with facultative Gram-negatives such as *Proteus*.

The compound is also fungistatic. When sixteen molds and yeasts, three of which were non-pathogenic, were tested on Sabouraud's medium containing 0.25% phenylethyl alcohol, only one, *Candida albicans*, proved resistant to phenylethyl alcohol. These fungi were streaked on the medium and incubated seven to fourteen days at 30°.

As has been reported (1), phenylethyl alcohol is also effective as an aerosol. Portions of 5 ml. each of 2.5 and 3.5% acetone solutions were placed on separate cardboard disks that had been cut and fitted in an aluminum Petri dish lid. After the acetone had evaporated (about twenty minutes), the lids were placed over sterile plates containing blood agar which had been streaked with a mixed culture of *Streptococcus* C-203 and *P. mirabilis* J2. After incubation for twenty-four and forty-eight hours, both concentrations produced well-isolated colonies of *Streptococcus* and *Proteus*. *Proteus* did not spread even when the lid was allowed to dry in the air for a long period of time. Control plates covered with acetone-soaked and dried lids were completely overgrown with *Proteus* within twenty-four hours. For best results with the aerosol type of test when blood plates are being used, incubation for only twenty-four hours is recommended; longer length of exposure to aerosol darkens the blood.

Organisms grown on basic media containing phenylethyl alcohol do not appear to change genetically. When they are transferred from the primary isolation on these media to a regular base medium, all the typical forms appear, e. g., *Proteus* spreads and the hemolysis of streptococci returns to normal expectancy.

Our results have led us to believe, after several trials, that 0.25% phenylethyl alcohol in infusion or trypticase-soy agar is a practical isolation medium. The inhibitor may be added to the boiled agar just before autoclaving. After the medium is sterilized, it may be stored in the refrigerator in tubes, flasks, or poured blood agar plates. There is a very gradual loss of inhibitor from the media during storage, particularly in containers with small surface exposure. Several days' supply may be prepared at a time but the storage period should not exceed a day or two when plates are poured, since changes in hemolysis may occur.

Although we have used freshly prepared medium in the major part of our work, a dehydrated form has been prepared commercially.[2]

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[2] Baltimore Biological Laboratory, 1640 Gorsuch Avenue, Baltimore, Md.

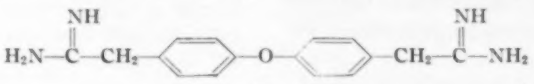
Symmetrical Bifunctional Analogs of Pharmacodynamically Active Substances*

By GEORGE P. HAGER and WEI-CHIN LIU†‡

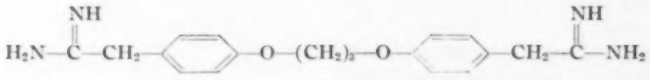
A continuation of a previously reported study on the use of chloromethylation products in the preparation of substances of pharmacologic interest has resulted in the preparation of the following amidines: 4,4′-bis(amidinomethyl)-diphenyl ether, 1,3-bis(4-amidinomethylphenoxy)-propane, and 1,5-bis(4-amidinomethylphenoxy)-pentane. The following were also prepared: the bifunctional aminoether, 4,4′-bis(2-diethylaminoethoxymethyl)-diphenyl ether; the bifunctional amine, 4,4′-bis(2-aminoethyl)-diphenyl ether; and the bifunctional imidazoline, 4,4′-bis(2-methylene-2-imidazoline)-diphenyl ether. The hydrochlorides of all the compounds prepared are being tested for pharmacologic evaluation.

IN CONSIDERING the relationship of structure to certain types of pharmacodynamic activity, it appears that a substance with a symmetrical disposition of similar characteristic functional groups possesses a higher order of activity than a related substance having a single functional group. It is possible, of course, that such a multiplicity of functional groups affects the pharmacodynamic properties of the molecule in a fundamental qualitative manner, a coordinated action of both functional groups of a bifunctional compound being necessary for production of a pharmacodynamic effect that is not characteristic of a related compound with only one functional group. Such is probably true of those compounds which have a pharmacodynamic effect that depends on some optimum intramolecular separation of the functional groups, e. g., chemotherapeutic diamidines (1), estrogenic phenols (2), curariform quaternary ammonium salts (3), etc. The possibility is intriguing that a symmetrical bifunctional analog of a pharmacodynamically active substance possessing only one functional group may resemble the latter qualitatively in its pharmacodynamic effects yet possess greater activity. If equimolecular concentrations of bifunctional and unifunctional substances at a given site of action could be achieved, the pharmacodynamic effect of the former might exceed that of the latter, provided each functional group performed as an independent unit. Without a doubt, consideration must be given eventually to the physical properties of the bifunctional analog especially as they affect such factors as absorption, distribution, etc.

Several diamidines have been prepared from bis(chloromethyl)-substituted aromatic hydrocarbons (naphthalene, biphenyl, and bibenzyl), and their antibacterial action on *Mycobacterium tuberculosis* $H_{37}Rv$, acute toxicity, and other biological properties have been reported (4). The structural relationship of these substances to stilbamidine was pointed out. This series has been extended to include compounds that are related isomerically or homologously to



(I)



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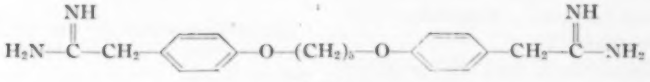

(III)

propamidine and pentamidine, *viz.*, 4,4′-bis(amidinomethyl)-diphenyl ether (I), 1,3-bis(4-amidinomethylphenoxy)-propane (II), and 1,5-bis(4-amidinomethylphenoxy)-pentane (III). The intermediate chloromethyl derivatives used

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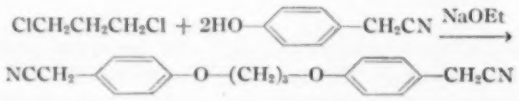
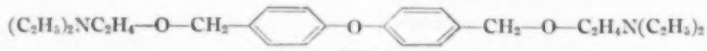
‡ The authors wish to acknowledge with appreciation the generous support given this work by Bristol Laboratories, Inc.

in the preparation of the amidines were prepared by a modification of the method previously described (5) for one of them, 4,4′-bis(chloromethyl)-diphenyl ether. The chloromethyl intermediates were converted to the corresponding diamidines by way of the nitriles and imido esters. The nitriles are intermediates in a number of procedures for the preparation of amidines (6) and, in the case of the diphenoxypropane and diphenoxypentane derivatives, were prepared more conveniently by the route indicated in the following equation:



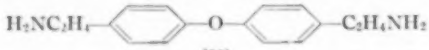
4,4′ - Bis(2 - diethylaminoethoxymethyl) - diphenyl ether (IV) was prepared in order to study the effect of the aryloxide grouping on the activity of the aralkyl ethers of amino alcohols



(IV)

previously reported (4), *viz.*, 4,4′-bis(2-diethylaminoethoxymethyl)-dibenzyl and the corresponding diphenyl derivative.

The dinitriles mentioned above also serve as intermediates for the preparation of bifunctional analogs of the sympathomimetic 2-phenylethylamines. 4,4′-Bis(2-aminoethyl)-diphenyl ether (V) was prepared by hydrogenation of the corresponding dinitrile, and was considered especially interesting in this possible series of compounds because of the probable contribution of the oxide linkage to the pharmacodynamic properties of the substance.

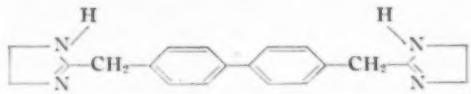


(V)

Compound V and the other possible members of this series also bear a structural relationship to various substances which have been found to possess antithyroid activity, especially 4,4′-diaminodiphenylmethane (7).

Bifunctional imidazolines may be prepared from the intermediate dinitriles or the corresponding esters by application of known methods (8). Compounds of this type which have been previously reported include 4,4′-bis(2-imidazolinel)-diphenyl (9) and 4,4′-bis(2-methylene-2-imidazoline)-diphenyl (10). The latter compound (VI) is included in a list of substances that were tested for histaminolytic activity. 4,4′-Bis(2-methylene-2-imidazoline)-diphenyl ether (VII), prepared in the course of this investigation, resembles compound VI from a structural standpoint. Both of these compounds are, in a sense, bifunctional analogs of the adrenergic-blocking agent, Priscoline (VIII).

Compound VII was tested[1] for its pharmacodynamic effect on the gastrointestinal tract, salivary glands, and cardiovascular system. It produced a depressor response on the blood pressure in doses of 0.5 to 10.0 mg./Kg. and had a variable effect in slightly reducing the pressor response to epinephrine. In a current continuation of this work the other members of the series of imidazoline derivatives are being prepared, as well as a parallel series of theophylline derivatives of which compound IX is a member.



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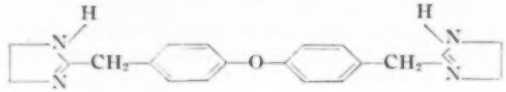

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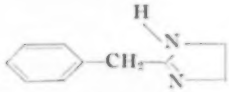


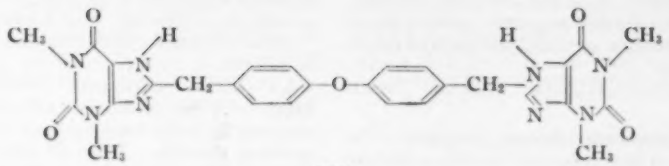
(VIII)

EXPERIMENTAL

All melting points are uncorrected and were observed by use of a Fisher-Johns melting point apparatus.

4,4′-Bis(chloromethyl)-diphenyl Ether.—The method described by Tomita and Kimura (5) was employed with some modifications. A mixture of diphenyl ether (10 Gm., 0.059 mole), glacial acetic

[1] The authors acknowledge with sincere appreciation the cooperation of Dr. H. L. Dickison, Bristol Laboratories, Inc., in obtaining the pharmacologic results herein reported.



(IX)

acid (15 Gm.), 40% formaldehyde solution (15 Gm.), and 85% phosphoric acid (13.7 Gm.) was stirred for five hours while a rapid stream of dry hydrogen chloride was introduced. The reaction mixture was poured into 300 cc. of ice water and extracted with ether. The ether solution was washed with 20% sodium carbonate solution, dried with anhydrous sodium sulfate, and concentrated by complete distillation of the ether. The residual oil was distilled under reduced pressure and the fraction distilling at 165–175°, 1.5 mm. pressure, was collected, allowed to stand until solid, and recrystallized from Skellysolve B or 95% ethanol. A yield of 6.8 Gm. (42.7%) of 4,4′-bis(chloromethyl)-diphenyl ether, m. p. 62–64°, reported m. p. 61–63° (5), was obtained.

1,3-Bis(4-chloromethylphenoxy)-propane.—This compound was prepared from 1,3-diphenoxypropane by essentially the same procedure as that described for 4,4′-bis(chloromethyl)-diphenyl ether, except that petroleum ether (200 cc. for 0.2 mole of 1,3-diphenoxypropane) was added to the reaction mixture which was treated with dry hydrogen chloride with stirring for eight hours at 90–95°.

When the reaction mixture was treated with ice water, a solid was obtained. This was powdered, washed thoroughly with petroleum ether and with water, and recrystallized from carbon tetrachloride. 1,3-Bis(4-chloromethylphenoxy)-propane, a white powder, m. p. 126–127°, was obtained in a yield of 35%.

Anal.—Calcd. for $C_{17}H_{18}Cl_2O_2$: Cl, 21.80. Found: Cl, 22.05.

1,5-Bis(4-chloromethylphenoxy)-pentane.—This compound was prepared from 1,5-diphenoxypentane (11) by essentially the same procedure as that described for 4,4′-bis(chloromethyl)-diphenyl ether, except that the reaction mixture was treated with dry hydrogen chloride at 90–95°.

When the reaction mixture was treated with ice water, a pasty mass resulted. This was washed with 10% sodium carbonate solution and with water, dissolved in warm Skellysolve B, and filtered. The crystalline material from the chilled filtrate was recrystallized twice from Skellysolve B. 1,5-Bis(4-chloromethylphenoxy)-pentane, m. p. 65.5 to 66.5°, was obtained in 22% yield.

Anal.—Calcd. for $C_{19}H_{22}Cl_2O_2$: Cl, 20.07. Found: Cl, 20.06.

4,4′-Bis(cyanomethyl)-diphenyl Ether.—This compound was prepared from 4,4′-bis(chloromethyl)-diphenyl ether by treatment with sodium cyanide in diluted alcohol and purification of the product by a method involving distillation and recrystallization according to the directions of Tomita and Kimura (5).

1,3-Bis(4-cyanomethylphenoxy)-propane.—*Method I:* The compound was prepared from 1,3-bis(4-chloromethylphenoxy)-propane by treatment with sodium cyanide, using the procedure previously described for the diphenyl ether derivative (5). The reaction appeared to be complete at the end of four hours and the solvent was removed by distillation under reduced pressure. The residue was washed with water and recrystallized from ethanol, using charcoal. 1,3-Bis(4-cyanomethylphenoxy)-propane, white crystals, m. p. 112–113°, was obtained in 41% yield.

Method II: 1,3-Dichloropropane (11.3 Gm., 0.1 mole) was treated with a mixture of sodium ethoxide (13.6 Gm., 0.2 mole), *p*-hydroxyphenylacetonitrile (26.6 Gm., 0.2 mole), and absolute ethanol (270 cc.), and the mixture was stirred under reflux for thirty hours. After cooling the reaction mixture, the crystalline material obtained by filtration was washed with water and recrystallized from 95% ethanol, using charcoal. 1,3-Bis(4-cyanomethylphenoxy)-propane, white crystals, m. p. 113–114°, was obtained in 60% yield.

Anal.—Calcd. for $C_{19}H_{18}N_2O_2$: N, 9.15. Found: N, 9.06.

1,5-Bis(4-cyanomethylphenoxy)-pentane.—*Method I:* The compound was prepared from 1,5-bis(4-chloromethylphenoxy)-pentane by treatment with sodium cyanide and purification of the product as described for 1,3-bis(4-cyanomethylphenoxy)-propane. 1,5-Bis(4-cyanomethylphenoxy)-pentane, m. p. 93–94°, white crystals, was obtained in 45% yield.

Method II: The compound was prepared from 1,5-dichloropentane as directed in the preparation of 1,3-bis(4-cyanomethylphenoxy)-propane by Method II. 1,5-Bis(4-cyanomethylphenoxy)-pentane, white crystals, m. p. 93–94°, was obtained in 55% yield.

Anal.—Calcd. for $C_{21}H_{22}N_2O_2$: N, 8.38. Found: N, 8.16.

4,4′-Bis(amidinomethyl)-diphenyl Ether.—Dry hydrogen chloride was passed into a solution of 15 Gm. (0.06 mole) of thoroughly dried 4,4′-bis(cyanomethyl)-diphenyl ether in a mixture of 10 cc. of absolute ethanol and 20 cc. of nitrobenzene, contained in a 1-L. tared suction flask surrounded by a freezing mixture, until an increase in weight of 5.5 Gm. (0.15 mole) was obtained. The flask was tightly stoppered and allowed to stand at room temperature for seven days. During this time, part of the mixture set to a solid mass. The crystalline imidoester hydrochloride was filtered, placed in a sealed tube with 100 cc. of 14% ethanolic ammonia, and shaken for five hours at 35–45°. The resulting solution was concentrated to one-half of its original volume by distillation of a part of the solvent, decolorized with charcoal, filtered, and treated while warm with anhydrous ether until it became cloudy. The white crystalline solid which separated was recrystallized from a mixture of absolute alcohol and ether, and 5.5 Gm. (27%) of 4,4′-bis(amidinomethyl)-diphenyl

ether dihydrochloride, m. p. 292–294°, was obtained. The free amidine, obtained as a white solid by treating the dihydrochloride with 10% sodium hydroxide, melted at 155–156°.

Anal.—Calcd. for $C_{16}H_{18}N_4O_2$: N, 19.85. Found: N, 19.32.

1,3-Bis(4-amidinomethylphenoxy)-propane.—The dihydrochloride of this compound was obtained from 1,3-bis(4-cyanomethylphenoxy)-propane as directed in the preparation of the diphenyl ether analog. By this means, a 28% yield of the compound was obtained as a white solid, m. p. 288–290° (dec.). The free amidine, prepared from the dihydrochloride, was obtained as a white solid, m. p. 215–217°.

Anal.—Calcd. for $C_{19}H_{24}N_4O_2$: N, 16.46. Found: N, 16.30.

1,5-Bis(4-amidinomethylphenoxy)-pentane.—The dihydrochloride of this compound was obtained from 1,5-bis(4-cyanomethylphenoxy)-pentane as directed in the preparation of the diphenyl ether analog. By this means, a 26% yield of the compound, a white crystalline solid, m. p. 295–297° (dec.), was obtained. The free amidine, prepared from the dihydrochloride, was obtained as a white solid, m. p. 166–167°.

Anal.—Calcd. for $C_{21}H_{28}N_4O_2$: N, 15.21. Found: N, 15.16.

4,4′-Bis(2-diethylaminoethoxymethyl)-diphenyl Ether Dihydrochloride.—4,4′-Bis(chloromethyl)-diphenyl ether (10 Gm., 0.037 mole) and sodium diethylaminoethoxide (10.4 Gm., 0.074 mole) were suspended in 100 cc. of dry toluene and the mixture was refluxed for twelve hours. The toluene was removed by distillation under reduced pressure. The residue was treated with 20 cc. of 10% sodium hydroxide and extracted with ether. The ether solution was extracted with 10% hydrochloric acid. The hydrochloric acid extract was made alkaline with sodium hydroxide pellets and extracted with ether. The ether solution was dried, using anhydrous sodium sulfate, and concentrated by distilling the ether under reduced pressure. The residual oil was distilled under reduced pressure and the fraction collected at 230–235°, 2 mm. pressure, was taken up in absolute ethanol previously saturated with dry hydrogen chloride. When the solution was treated with dry ether, a hygroscopic precipitate formed and was recrystallized from acetone. By this means, 11 Gm. (59%) of 4,4′-bis(2-diethylaminoethoxymethyl)-diphenyl ether dihydrochloride, white crystals, m. p. 160–161°, was obtained.

Anal.—Calcd. for $C_{28}H_{42}Cl_2N_2O_3$: N, 5.59. Found: N, 5.78.

4,4′-Bis(2-aminoethyl)-diphenyl Ether Dihydrochloride.—A mixture of 6.2 Gm. (0.025 mole) of 4,4′-bis(cyanomethyl)-diphenyl ether, 3.1 Gm. of Raney nickel, and 75 cc. of 15% methanolic ammonia was shaken for three hours at 60–65° under 1,100 p. s. i. hydrogen pressure. The reaction mixture was filtered through a layer of charcoal and concentrated by distillation of the methanol under reduced pressure. The residue was distilled under reduced pressure, and the fraction collected at 170–175°, 3 mm. pressure, was taken up in a solution of dry hydrogen chloride in absolute ethanol. By precipitation of the solution with ether and recrystallization of the precipitate from a mixture of alcohol and ether, 6.5 Gm. (79%) of 4,4′-bis(2-aminoethyl)-diphenyl ether dihydrochloride, a white crystalline powder, m. p. 295–297° (dec.), was obtained.

Anal.—Calcd. for $C_{16}H_{22}Cl_2N_2O$: N, 8.51. Found: N, 8.51.

4,4′-Bis(2-methylene-2-imidazoline)-diphenyl Ether.—4,4′-Bis(carboxymethyl)-diphenyl ether was obtained in crude form by hydrolysis of the corresponding dinitrile. The acid was esterified by refluxing a mixture of 6 Gm. (0.021 mole) of the crude acid with 120 cc. of absolute ethanol containing 3 Gm. of sulfuric acid. The resulting diethyl ester was isolated in the usual way and purified by distillation under reduced pressure, $b._3$, 205–208°; yield, 70%.

A mixture of 4 Gm. (0.012 mole) of 4,4′-bis(carboethoxymethyl)-diphenyl ether and 5.8 Gm. (0.096 mole) of anhydrous ethylenediamine was heated in a sealed tube on a steam bath for thirty-six hours. The excess ethylenediamine was removed by distillation under reduced pressure. The residue was recrystallized from dry xylene and 2.8 Gm. (72%) of 4,4′-bis(2-methylene-2-imidazoline)-diphenyl ether, a white solid, m. p. 163–164°, was obtained.

Anal.—Calcd. for $C_{20}H_{22}N_4O$: N, 16.76. Found: N, 16.58.

The dihydrochloride, a white solid, m. p. 212–213°, was prepared by treating a solution of the imidazoline in dry xylene with dry hydrogen chloride.

SUMMARY

1. In a further study of diamidines obtained from the products of bis(chloromethylation) of hydrocarbons and phenolic ethers, the following compounds which are isomerically and homologously related to propamidine and stilbamidine have been prepared: 4,4′-bis(amidinomethyl)-diphenyl ether, 1,3-bis(4-amidinomethylphenoxy)-propane, and 1,5-bis(4-amidinomethylphenoxy)-pentane.

2. One of the intermediates, 4,4′-bis(chloromethyl)-diphenyl ether, was also converted to a bifunctional amino-ether [4,4′-bis(2-diethylamino ethoxymethyl)-diphenyl ether], a bifunctional analog of 2-phenylethylamine [4,4′-bis(2-aminoethyl)-diphenyl ether], and a bifunctional analog of Priscoline [4,4′-bis(2-methylene-2-imidazoline)-diphenyl ether].

3. Pharmacologic studies revealed that 4,4′-bis(2-methylene-2-imidazoline)-diphenyl ether produces a lowering of the blood pressure and appears to decrease the pressor response to epinephrine to some extent. Current investigations have the objective of producing other members of this series of imidazolines and also a parallel series of theophylline derivatives.

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Validity of Vein Islet Numbers in Species Distinction*,†

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An evaluation of vein islet procedures is presented, with recommendations for improving the visibility of the ultimate vein branches by treating with phloroglucinol.

A critical study of vein islet procedures was undertaken with the hope of eventually establishing vein islet numbers for the more important official leaf drugs and especially those in which questions of species identification may arise.

A vein islet, according to Eames and MacDaniels (1), is the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. Since this area is a small decimal, making a comparison of results difficult, Levin (2) found it more practical to express the results in terms of the number of vein islets per square millimeter. He termed this the "vein islet number." All results obtained in this investigation will be expressed in terms of the vein islet number, as defined by Levin, corrected to the nearest half.

Early investigators in the field of vein islet determination as a method of species identification differed widely in both methods employed and results obtained. Ensign (3) disproved much of the earlier work done by Benedict (4) as to the accuracy of the methods employed. He showed that from 17 to 62 per cent of the vein islets of 38 leaves were not apparent in the uncleared leaves. Thus, any results obtained without first removing the interfering chlorophyll would prove to be very unreliable.

Smith, *et al.* (5), reported that all or almost all of the cells of a typical leaf enlarge and mature more or less simultaneously, the cells having been formed relatively early with cell division then ceasing. These early cell divisions occur chiefly in the apex of the leaf primordium, but later divisions occur throughout until the leaf has assumed the form and shape of a mature leaf. The increase in size is due in part to the enlargement and maturation of cells already present and not primarily to an increase in the number of cells followed by their enlargement. On this premise, the individual cells of the conducting tissue, originally present in the immature leaf, merely increase in size during the period of leaf expansion. Few or no additional cells are developed in the central portion of the lamina, the area usually used for vein islet determinations.

If this is the case, the vein islet number for a large mature leaf of any given species should be less per square millimeter than that of a smaller leaf.

This variation in vein islet number in relation to size of leaf may not be too apparent in the smaller leaved plants such as buchu, senna, and coca, but in the larger leaved plants such as the *Daturas* and digitalis, the results presented in Table I substantiate the opinions of Smith, *et al.* (5).

It is evident that the variation in the case of digitalis is not as pronounced as in the *Daturas*. This is probably due to the vein islet numbers in digitalis being uniformly lower than those in *Datura*.

Turrell (6), working on the internal surface of large and small leaves of alfalfa, reported that the mean length and the mean width of a mesophyll surrounded by minor veins (vein islets) was larger in the large leaves than in the small leaves.

Benedict (4) also stated that the venation of leaves of older plants differed from the leaf vena-

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TABLE I.—VARIATION IN VEIN ISLET NUMBER WITH SIZE OF LEAF

Size, Cm.	Vein Islet No.
Datura metel	
3.2 x 6.6	33
7.7 x 14.5	19
Digitalis purpurea	
1.6 x 3.9[a]	7.5
4.2 x 12[a]	5.0
1.2 x 3.7[a]	6.5
5.5 x 8.5[a]	5.5
2.7 x 6.2[a]	7.5
5.3 x 10.9[a]	5.0
10.2 x 38[b]	2
3.5 x 10[b]	3
1.3 x 4.2[b]	4

[a] These leaves were obtained from the Herbarium of the Columbia University College of Pharmacy. Each pair of leaves grouped together was obtained from the same plant.
[b] This material was a fresh specimen supplied by S. B. Penick Co., New York.

tion of younger plants. Ensign (3), using leaves of *Vitis vulpina* from plants of different ages, could not show a single instance in which venation could be used to determine relative ages of plants. Therefore, Ensign concluded that the vein islet number remains constant within a given species regardless of age, and this was accepted by Levin. However, this number may show slight variations within a narrow range.

Other investigators such as Benedict, Schuster, Tellefsen, and Zalenski, as reported by Wylie (7), have shown that marked differences in venation may occur within a given species and even among leaves of the same plant. As has been reported, the vein islet number does not always appear as a relatively constant figure for any species, as it may vary with existing environmental conditions during early growth, with the position of the leaf insertion on the same axis, and also (as shown here) with the size of the leaf under consideration.

Other aspects of this problem are presented by Wylie (7), who studied the relations between tissue organization and vein islet distribution. He reported that cells primarily concerned with the development of the minor veins retain the capacity for normal cell division longer than the other cellular elements. This aids the leaf in modifying its vein pattern to the type of mesophyll being developed. This development is more or less determined by specific local conditions such as intensity of illumination, water supply, and other factors. Additional investigation seems to be warranted to determine the extent of the change in the vein islet pattern, even though these conditions may not alter the vein islet number to any considerable degree. Wylie (8) also found that the spread of major and minor veins, including veinlets, resulted in a fairly uniform distribution of conductive strands for a given plant, varying somewhat with the height of leaf insertion and in a lesser degree with different parts of the blade.

In view of these diverse opinions it appeared desirable to confirm Levin's work. The leaf of *Erythroxylon coca* was selected because it cleared easily in a hot aqueous solution of chloral hydrate (50–20), and the ultimate ramifications of the veins into the mesophyll were always discernible without any further treatment.

The Levin investigation also included vein islet counts on *Barosma betulina*, *Cassia angustifolia*, and *Digitalis purpurea*, each of which offers greater difficulty than *Erythroxylon coca* in handling and counting. These three leaves were reexamined and the results of this check verify the counts suggested by Levin.

EXPERIMENTAL

Procedures in Vein Islet Determinations.—A Leitz microprojector, giving magnifications of 10× to 15×, was substituted for the camera lucida used by Levin, and suitable areas of the specimen were projected on a paper ruled into areas representing 4 square millimeters each. Depending upon the size and shape of the leaf, projected areas of 1 x 4 mm. and 2 x 2 mm. were used, but the final results were always expressed per square millimeter. In making the counts, only those vein islets were included that came entirely within the ruled area on two adjacent sides, and on the other two sides they were included if only part of the vein islet entered into the ruled area.

Counts were made from the marginal, apical, and basal portions of the lamina, but such counts showed too great a variation to be of service. Greater uniformity was noted in preparations from the central portion of the lamina or the area equidistant from apex and base as well as margin and midrib. All subsequent counts were determined from this central portion of the lamina.

Levin found that most leaves could be sufficiently cleared by the use of a hot aqueous solution of chloral hydrate (50–20). This appears to be one of the most effective clearing agents. The activity is due to the solvent action of the chloral hydrate on the various plant constituents. Not only will chloral hydrate dissolve resins, starches, proteins, and chlorophyll, but it also causes some expansion of any shrunken cells.

For those leaves that did not satisfactorily respond to this treatment, Levin stained the leaf with safranin (a saturated solution of safranin diluted with an equal volume of alcohol) for forty-eight hours, removing the excess stain with alcohol, and mounting in glycerin. This procedure was not successful for many leaves and he proposed still another technique in which the leaves were soaked in water and the epidermis was stripped off. The leaf was then treated with a solution of chlorinated soda until colorless (from six to twenty-four hours), followed by

washing with distilled water to remove the bleaching solution. If the leaves contained calcium oxalate crystals, they were then soaked in a 10% solution of hydrochloric acid to dissolve out the interfering crystals and obtain the finer details of the venation. Finally the leaves were soaked in an aqueous chloral hydrate solution and heated on a water bath until transparent.

In reviewing Levin's results, it became apparent that numerous discrepancies existed. The leaves of *Barosma betulina* and *Barosma serratifolia* gave vein islet counts within the limits proposed by Levin only after the epidermis was removed following a preliminary soaking in water. This procedure is not possible with the leaves of many other genera. Examination of *Digitalis purpurea* yielded counts within the limits Levin reported, while *Erythoxylon coca* at first showed a low count of 7–8. Counts within the limits reported by Levin were obtained only by shortening the chlorination time or by prolonged treatment with a hot solution of chloral hydrate.

Examination of senna leaflets by the Levin technique yielded variable results and for the most part the counts were very low, averaging 2–10. It was obvious that many of the tertiary veins were not visible. They were, in fact, completely obliterated. Results are shown in Table II.

A further modification of the clearing methods used by Levin is mentioned by Baden (9) in his essay on "Taxonomy of Commercial Sages." In this modification, the leaves are soaked, until pliable, in water containing a small quantity of a wetting agent to aid in penetrating the epidermis. A solution of sodium hypochlorite was used full strength as a bleaching agent. The time required for this step was about three hours. He cautioned that care must be exercised, as the leaves would completely disintegrate if immersed too long in the bleach. After bleaching, the leaves are washed in distilled water and placed in a hot saturated aqueous chloral hydrate solution on a water bath until the specimens are clear.

Since this method did not yield reliable results with the senna leaflets, it appeared possible that the bleaching process was responsible for the lack of detail in the finer vein branches.

TABLE II.—AVERAGE VEIN ISLET COUNTS

Leaf	Av. Vein Islet Counts
Barosma betulina	10–15.5
Cassia angustifolia	21
Erythroxylon coca	8–12
Digitalis purpurea	2– 5.5

Lignification in Relation to Vein Islets.—Obviously the object of all these clearing methods is to render the ultimate vein branches clearly visible with the least damage to these delicate structures. While chloral hydrate treatment sufficiently clears some specimens, it must be supplemented by bleaching agents in others, and the latter are more or less destructive to the material. Levin suggested safranin staining, but owing to the small amount of lignin in these vein branchlets, it is difficult to secure satisfactory definition by this means. Therefore attention was directed to the matter of lignin occurrence in these branchlets and the possibility of a staining procedure for increasing their visibility. This consideration necessarily involves the basic structure of these smaller veins. Holman and Robbins (10) summarized the situation as follows: "Although the vascular bundles of the petiole and large veins contain vessels, tracheids, sieve tubes, and companion cells, the vein endings consist only of tracheids, the vascular bundles being reduced generally at the very end to a single tracheid which is usually of the spiral type."

If at least one spiral vessel is presumably always present, it can be concluded that some quantity of lignin or ligno-cellulose is usually present. Perhaps this is the answer to why the smaller veins were obliterated, as any prolonged treatment with an oxidizing agent such as sodium hypochlorite will remove lignin from tissues and thus cause the smaller veins to disintegrate.

Many aspects of the chemistry of lignin are very obscure. This is partially due to the fact that its constitution remains undefined despite continued research (11). Because of the difficulty of isolating pure lignin from the other cell wall constituents, its chemical composition is not definitely known. On the other hand we have considerable information on its properties and behavior on treatment with several chemical reagents. Chief among these are its resistance toward strong acids, whereas alkaline solutions will cause lignin to pass readily into solution. Lignin is very unstable toward oxidizing agents such as the peroxides and hypochlorites, and such reactions, if allowed to go to completion, yield only the simplest degr .dation products such as the lower fatty acids and dibasic acids as oxalic and succinic.

In the presence of strong acids, lignin condenses with aldehydes and ketones, yielding insoluble products of the phenol-aldehyde resin type. The proposed method of staining the venation of leaves depends upon the fact that lignin has aldehydic or phenolic properties. The most sensitive test for determining the presence of traces of lignin is the treatment of the tissue with an alcoholic solution of phloroglucinol followed by concentrated hydrochloric acid. A bright red to red-violet color is formed. In the majority of cases the chemistry of the reaction is not clear. Owing to the varying proportions of lignin and ligno-cellulose in dried leaves, the red coloration may not always be too apparent, but a definite darkening of the fine vein branches is visible.

Since lignin will condense in strong acid solutions with aldehydic or ketonic substances yielding a dark, insoluble product, the best reagent appears to be phloroglucinol, which is ketonic in nature. Other phenols can be used, but due to the existing tautomerism displayed by phloroglucinol, they offer no special advantage over this compound.

The use of hydrochloric acid following the treatment with phloroglucinol increases the coloration and also aids in the removal of many of the interfering plant constituents such as calcium oxalate crystals.

Phloroglucinol is especially useful in dealing with dried materials where it is frequently difficult to obtain clear preparations with chloral hydrate even when supplemented by bleaching agents; the risk of damage by the latter is especially great. Some-

times it may be desirable to increase the intensity of this reaction. A suggested modification is to add a small quantity of xylose at the time the solution is acidified. This causes a disturbance in the reaction which is primarily thought to be due to the condensation of soluble fission products with the lignin, thereby increasing its weight. Lignin has been shown to condense readily in acid solutions with aldehydes or ketones. In the case of pentoses in strong acid solutions there is a slow production of furfuraldehyde by dehydration. If lignin is present, condensation with furfural occurs, leaving a black insoluble precipitate. This darkening will occur on the lignified smaller vein endings.

Proposed Staining Procedure.—(*a*) Soak leaves in a saturated solution of chloral hydrate and heat on a water bath until clear.

(*b*) Wash in distilled water and transfer leaves to a freshly prepared solution of phloroglucinol (500 mg. in 25 cc. of alcohol) for three to five minutes.

(*c*) Acidify with a 10% to concentrated hydrochloric acid solution and leave in solution long enough to dissolve out any interfering calcium oxalate crystals.

(*d*) Wash with distilled water and treat leaves again with a hot aqueous saturated solution of chloral hydrate on a water bath until clear. The leaves may be kept in this solution until ready for use.

(*e*) The prepared leaves are mounted in glycerin-alcohol-water (1:1:1) solution.

Suitable areas are projected with the aid of a Leitz microprojector and the vein islets are then traced on paper ruled in areas representing square millimeters.

Comments.—(*a*) If the leaves are small, the whole leaf is cleared and stained. For larger leaves, the central portion, as previously described, is used.

(*b*) Staining procedures (Step *b*) are used only if the smaller vein endings are not clearly discernible after clearing with a hot saturated solution of chloral hydrate.

(*c*) The concentration of the acid (Step *c*) will depend upon the condition of the leaves, as old dried leaves may not withstand the treatment with a concentrated acid.

Vein Islet Numbers of Datura Species.—Species identification is frequently of importance in the examination of stramonium leaves offered in drug commerce, as the National Formulary specifies *Datura stramonium* and *Datura tatula*. If the leaves are in an unbroken condition the detection of other species may not be too difficult, but more often the sample is in comminuted form. As *Datura* was not included in the Levin study, several species were examined to determine if the vein islet procedure would be helpful in species distinction (Table III).

Using either chloral hydrate as the clearing agent or the staining method previously described, vein islet numbers for several species of *Datura* leaves were determined. Most of the leaves used were obtained from Herbarium specimens. These were, in many cases, supplemented by stock supplied by S. B. Penick & Co. and J. L. Hopkins & Co.

It can be seen from these figures that the leaves of *D. stramonium* and *D. tatula* cannot be distinguished from each other by vein islet number. To distinguish them from *D. meteloides* and *D. metel* by this method is also difficult, if not impossible. If vein islet numbers are used as a diagnostic character in describing these and other species of *Datura*, it seems advisable to include the range of the counts, as variations and overlapping of these ranges occur, making a differentiation of most species quite difficult.

TABLE III.—SUMMARY OF VEIN ISLET NUMBERS IN GENUS DATURA

Species	V. I. Range	Av. V. I. No.
Stramonium	11–27	21
Tatula	10–33	21
Meteloides	15–46	27
Metel	17–50	23
Alba	12–17	14.5
Ferox	7–16	10.5
Quercifolia	14–22	18

SUMMARY

Vein islet number determinations appeared promising as an aid in species determinations, especially in working with fragments of leaves so frequently encountered in drug supplies.

The chief difficulty in technique is in clearing the specimen so that the ultimate vein branches will be clearly apparent.

A method is outlined for obtaining greater visibility of these ultimate vein branches by treatment with phloroglucinol.

When leaves of a species are of uniform size, the vein islet count may aid in species distinction, but there is considerable variability in range within a given species.

The many variables of position, environment, size, and other factors materially affect the utility of vein islet numbers in leaves of larger size than those included in Levin's study.

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Effect of Traumatic Shock and Exhaustion on Infection with Tetanus Spores*

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In view of the reported manifestation of latent tetanus infections in humans exposed to stress or shock, this subject was investigated experimentally by using mice. Latent tetanus infection was produced by the injection of washed tetanus spores under aseptic conditions. Standard shock was produced by the Noble-Collip method; standard degree of exhaustion was produced by employing the swim technique and by exposing the mice to low temperatures. Manifestation of the disease was not evidenced in any of these instances. On the other hand, active tetanus was induced by the subsequent injection of calcium chloride solutions. The relationship between shock, stress, and tetanus infection is discussed.

In 1892 Vaillard and Rouget (1) observed that if tetanus cultures were heated to 65–67° for half an hour to destroy the vegetative bacilli and the toxin, the remaining toxin-free spores could be injected in large numbers into a guinea pig without giving rise to the disease. Microscopic examination showed that the spores did not germinate in the tissues, but were rapidly taken up by the phagocytes, so that in two or three days they were completely ingested. Although the spores were rapidly ingested, they were not necessarily destroyed, but might remain latent within the cells, and spontaneously give rise to tetanus some months afterwards.

The same results (tetanus infection) could be obtained by inflicting a trauma at the site of injection, sufficient to cause tissue necrosis or effusion of blood, or by creating a simple fracture. In the damaged tissue the spores were able to germinate, whereas in clean aseptic wounds they were unable to do so. Other organisms in the wound, particularly aerobes, also permitted the germination of the spores. From these observations it appeared that toxin-free spores are innocuous, but that they can be activated after injuring the tissues or by a secondary infection.

Other workers (2, 3) showed that toxin-free spores were rendered pathogenic for guinea pigs by the simultaneous or later injection of staphylococci or tissue debilitants such as quinine or calcium chloride. These experiments revealed that the spores may survive in the tissues for some time. Tullock (4) in 1919 found the bacillus nineteen times in 100 soldiers examined for non-tetanus wounds. Further observations also showed that tetanus might not develop for weeks or months after the wound had healed, and might then appear suddenly after an operation or injury.

Recent investigations have revealed that many substances possessing a physiological and pharmacological effect upon the body such as shock substances [adenosine triphosphate, etc. (5–7)], adrenal hormones (8–10), and antihistamines, (11, 12), also increased the infectivity in certain infections. In view of the above and the knowledge that shock and trauma during injury on another part of the body in animal experiments and in human infections stimulated tetanus infection many weeks or months after the wound containing the spores had healed (3, 4), the question of the effect of standardized forms of stress and shock on latent tetanus infection in mice was investigated experimentally. This problem appeared pertinent because of the reported manifestation of latent tetanus infections in soldiers constantly exposed to stress and shock.

MATERIALS

The Noble-Collip shock drum was utilized to introduce standardized experimental shock in small animals by constantly tumbling them in a drum (16 in. in diameter) until a state of shock develops in the animals. In this technique, which is known as trauma shock, hemorrhaging and fracture occur only rarely. The mice, which were the experimental animals used, were anesthesized with ether for several minutes in an anesthesia jar, then quickly removed; their fore and hind legs were then bound with scotch tape to prevent moving in the drum, thereby causing an exhaustion effect rather than the desired shock effect. The drum was electrically whirled at 40 r. p. m.

The standardized stress technique consisted of placing mice in a large glass jar containing cold water at a temperature of 5° ± 2° which was kept approximately at a constant temperature by the addition of ice cubes. The mice were allowed to swim in the cold water until exhaustion occurred. They were then removed from the water and placed on sawdust to dry.

Swiss male mice (Webster strain) were used in all animal experiments. The animals were given

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Rockland Complete Rat Checkers and water *ad lib.* All animals were housed in air-conditioned quarters at 21°, in draft-free cages.

The spores of *Clostridium tetani* were prepared by growing the organisms in F. D. A. broth containing 10% dextrose (*p*H 7.4 to 7.6), under anaerobic conditions. The spores were collected by aseptic centrifugation, washed with sterile isotonic sodium chloride 3 times, and then heated to 80° for ten minutes to destroy vegetative forms and aerobic nonspore-forming contaminants. The spores were stored in the refrigerator at 4° until needed.

Sterile isotonic solution of sodium chloride (0.9%) U. S. P., herein referred to as "sterile isotonic sodium chloride," was used.

Syringes and needles were washed carefully with warm water, rinsed with distilled water, and then sterilized in the autoclave at 121.3° for at least thirty minutes.

EXPERIMENTAL

Attempts to Stimulate Latent Tetanus Spore Infection by Acute Shock

Technique.—Male Webster mice which had been injected one month earlier with tetanus spores and did not contract the infection were divided into 3 groups.

The first was a control group consisting of 12 mice which were observed daily to see if the disease would develop from latent spores in the tissues without external influence or stimulation.

Group 2 consisted of 40 male Webster mice (previously injected with tetanus spores) which did not contract the disease. The mice in groups of 3 were placed in a large glass desiccator jar which was filled with ice water (5° ± 2°) and treated by the standardized stress technique described above.

The test animals were listless after entering a state of shock. Consciousness was not completely lost, although the animals responded very sluggishly to any type of stimuli. The respiration approached apnea. This condition lasted several hours. On the following day the surviving mice returned to normal activity and seemed to show no ill effects from the previous day's ice bath treatment. The animals in the experimental group were immersed in the cold water bath every forty-eight hours for six weeks and observed every day for symptoms of tetanus infection.

A third group consisting of 40 male Webster mice injected one month earlier with tetanus spores was placed in a state of acute shock produced by means of the Noble-Collip shock drum. Five mice at a time were anesthesized in an ether jar, quickly removed, and their front and hind legs bound with scotch tape. This prevented the animals from running in the drum and developing fatigue rather than the desired shock effect. Each group of 5 mice was originally placed in the drum for five minutes or a total of 200 revolutions. As the mice became adapted to the shock effect they were exposed to longer shock periods up to twelve minutes. When exposed to time periods longer than this, many of them died. The symptoms of shock were similar to those observed in the cold water bath group. The mice were placed in the Noble-Collip drum every forty-eight hours and observed daily for symptoms of tetanus.

Findings.—None of the control mice developed tetanus during the six-week observation period.

None of the mice in the swimming stress group developed tetanus. Eleven of the 40 mice did not survive the experiment; however, the actual cause of their deaths could not be determined. The 29 mice which survived the experiment and did not contract tetanus by the stimulation of severe stress showed no harmful after-effects from the ice water treatment and were normal and healthy upon completion of the experiment. The average length of immersion time at the beginning of the experiment was three to five minutes and at the end of the six-week period from five to eight minutes. Thus the mice did not seem able to adapt themselves greatly to the severe shocking effect of the ice water bath.

In the third group subjected to the Noble-Collip drum, none of the 40 experimental mice showed signs of tetanus infection, although 18 animals died from unknown causes. None of the 18 developed tetanus symptoms. The mice developed significant resistance against acute shock caused by the Noble-Collip drum as compared to the lack of development of resistance in the cold-water bath group. The former group were able to adapt themselves to an increase from 200 revolutions of the drum to 480 revolutions. Increasing the exposure time beyond twelve minutes (480 revolutions) rapidly increased fatalities among the mice due to severe shock.

The Effect of Cold in Stimulating Latent Tetanus Spore Infection

Technique.—Forty male mice which had been injected one month earlier with tetanus spores and did not develop tetanus were divided into 4 groups of 10 mice each. Two groups of mice were placed in a refrigerator (temperature 1°), the other two groups in another refrigerator (temperature 4°). The mice were observed daily for a period of two weeks for symptoms of tetanus. Food and water were given *ad lib.*

Findings.—Although the mice were continually exposed to extreme cold for two weeks, none developed tetanus. However, 15 mice died from exposure and disease. Animals surviving the experiment were in very poor physical condition. Many of the mice had gangrenous feet and tails and lost weight, although normal amounts of food and water were consumed while the mice were in the refrigerator.

The Effect of Quinine Hydrochloride and 3% Calcium Chloride Solution in Stimulating Latent Tetanus Spore Infection

Technique.—Forty-three male Webster mice (25–30 Gm.), which had been injected one month earlier with tetanus spores in the right flank and did not contract the disease, were divided into 3 experimental groups.

The first group, consisting of 20 mice, received 300 mg./Kg. of quinine hydrochloride which was prepared in sterile distilled water; 0.3 ml. was injected subcutaneously into the right flank of each of the 20 mice.

The second group, consisting of 10 mice, received 0.3 ml. of 3% $CaCl_2$ solution subcutaneously in the right flank.

The third group of 13 mice received 0.3 ml. of 3% $CaCl_2$ solution subcutaneously in the left flank.

TABLE I.—THE EFFECT OF SUBCUTANEOUS INJECTIONS OF QUININE HYDROCHLORIDE AND 3% $CaCl_2$ SOLUTION IN STIMULATING LATENT TETANUS SPORE INJECTION

Experimental Group	No. of Mice Used	Death with Tetanus Symptoms	Survivors Exhibiting Tetanus Symptoms	No Symptoms	Death Cause Unknown
Quinine hydrochloride, 300 mg./Kg. in right flank where spores had been previously injected	20	0	0	19	1
3% $CaCl_2$ solution in right flank where spores had been previously injected	10	5	0	4	1
3% $CaCl_2$ solution in left flank where no spores had been injected	13	0	0	13	0

The 3 experimental groups were kept under observation for three weeks to detect symptoms of tetanus.

Findings.—Quinine hydrochloride injected into the right flank of mice, which had been injected with tetanus spores one month earlier, did not stimulate latent tetanus infection.

The injection of 3% $CaCl_2$ solution into the same area of the original spore injection did cause tetanus and death in 5 out of 10 mice, whereas injections of the same compound into the opposite flank from the original tetanus spore injections did not stimulate latent tetanus infection (see Table I).

The 3% $CaCl_2$ solution injections caused severe tissue necrosis in both calcium chloride groups.

DISCUSSION

Although numerous workers have claimed that dormant tetanus spores may be stimulated to activity long after the original introduction of the spores into the tissues (1–3), it appears that the sudden release of acute shock substances in mice subjected to standardized shock or stress methods such as swimming in a cold water bath or by the Noble-Collip shock drum cannot cause the stimulation and germination of tetanus spores in mice which had been injected with spores one month previous to the shock effects. This failure to cause tetanus may be due to several factors. First, the spores may have been completely phagocytized and removed from the site of injection. That this is not true was demonstrated by the stimulating of tetanus spore infection by the injection of 3% $CaCl_2$ solution at the original spore injection site. Second, the shock effect may not have been great enough to cause a general ischemia in the area of the spore injection; thus a condition of an anaerobiosis would not develop with subsequent spore germination. The third and possibly the most important reason is that mice are much more resistant to tetanus infection than guinea pigs. Bullock, *et al.* (3), found that cold did not stimulate latent tetanus spores in mice, whereas Tullock (4) claimed that cold stimulated spore infection in guinea pigs. Francis (2) could not stimulate latent tetanus spore infection in mice with quinine injections but the same procedure in guinea pigs resulted in infection. Ambrus, *et al.* (13), and others have demonstrated that the guinea pig is much less resistant to equivalent doses of shock substances than are mice.

Injections of quinine hydrochloride at the site of earlier spore injection and 3% $CaCl_2$ solution at a site distant from the spore injection did not cause tetanus, possibly for the same reasons as mentioned above.

SUMMARY AND CONCLUSIONS

1. Mice previously injected with tetanus spores without contracting the disease were subjected to acute shock or stress by swimming in a cold water bath or by the Noble-Collip shock drum. None of the mice developed tetanus.

2. The effect of continual cold in sensitizing mice to latent tetanus spore infection was investigated by placing mice previously injected with tetanus spores in cages into refrigerators at 1° and 4° for two weeks. It was observed that continued cold did not increase susceptibility of mice to latent tetanus spore infection.

3. Quinine hydrochloride (300 mg./Kg.) injected into the original spore injection area one month later did not stimulate latent tetanus infection in mice, whereas a 3 per cent calcium chloride solution injection into the original spore injection site stimulated the infection in five of ten test mice.

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Ultraviolet Spectrophotometric Estimation of Khellin in Human Blood*

By FELIX G. SOLONI and JUAN F. MÁRQUEZ†

A spectrophotometric method is described for the quantitative estimation of khellin in blood. Chromatographic adsorption of impurities from alcoholic solutions is employed. The absorption curves of khellin in alcohol have been determined and the molar extinction coefficient in the region of 220–350 mμ has been calculated. The ultraviolet absorption of khellin provides a quantitative method for its determination in alcoholic solutions. The method described permits the performance of a series of determinations in the same patient, and has the advantage of utilizing only 2 ml. of blood and very small amounts of reagents. The procedure requires only fifteen minutes to perform.

MUSTAPHA (1) in 1897 obtained a crystalline product, which he called khellin, by means of alcoholic and ether extraction of a West Mediterranean coast plant called Khella in Arabic (*Ammi visnaga* L.) These crystals were composed of a group of substances from which three were isolated by Späth and Grüber (2–4). All of these substances belong to the group of furanochromones. The most powerful, which is still called khellin, increases coronary circulation without increasing arterial tension.

The methods described by previous workers (5–9) were found by us to be unsuitable for the measurement of khellin in human blood. A method has been devised which permits quantitative determinations of khellin in quantities of 1–2 ml. of blood, making it possible to repeat tests in the same patient (see Table I).

EXPERIMENTAL

Determination of the Molar Extinction Coefficient

By use of standard solutions of 12, 10, 7.5, 5, 2.5, and 1 μg./ml., the average value of the molar extinction coefficient was determined in silica cells of 1.004 cm., taking 260.096 as the molecular weight of khellin.

The figures obtained have been plotted in Fig. 1. The ultraviolet spectrum of khellin shows maximum absorption at 250 mμ. The location of this maximum has been confirmed by observing the absorption at 0.5-mμ intervals between 245 and 255 mμ.

Calibration Curve

For plotting a calibration curve, several standard khellin solutions were prepared with 96% alcohol. A sample of 0.2500 Gm. of khellin (m. p. 153.5 to 154° C.; 95% purity)[1] was diluted at 20° to a volume of 250 ml.

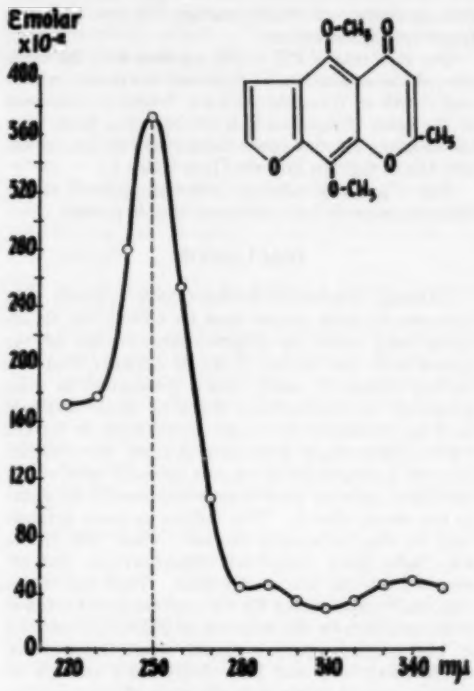

Fig. 1.—Khellin (alcohol).

One milliliter of this solution was diluted to 10 ml. in a volumetric flask at 20°, 96% alcohol being used to make this and subsequent dilutions. This new solution contained 100 μg./ml. From this solution, aliquots of 1.20 ml., 1.00 ml., 0.75 ml., 0.50 ml., and 0.25 ml. were taken and diluted individually to 10 ml. in volumetric flasks at 20°, giving concentrations of 12.0, 10.0, 7.5, 5.0, and 2.5 μg./ml., respectively. From the solution containing 10 μg./ml., a 1-ml. aliquot was diluted to 10 ml., or 1 μg./ml. The curves of the ultraviolet absorption from each of these solutions were used, using 96% alcohol as a blank.

The law of Bouguer and Beer is borne out in maximum as well as in other wave lengths as can be seen in Fig. 2. It is obvious that the calibration curves

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[1] It must be pointed out that the percentage of purity of the khellin used (95%) is given on the basis of the statement of Laboratorios A. Murai who supplied the product.

must be adjusted to the experimental conditions of each laboratory.

The following slit widths were used: initially, 1.8 mm. (Nominal Band Width: 2.6 mμ); at 250 mμ, 0.49 mm. (N. B. W., 1.08 mμ); at 290 mμ, 0.35 mm. (N. B. W., 1.26 mμ); at 310 mμ, 0.32 mm. (N. B. W., 1.44 mμ); at 340 mμ, 0.284 mm. (N. B. W., 1.73 mμ); ending with a slit of 0.281 mm. (N. B. W., 1.80 mμ). The temperature was maintained at 22°.

It is not necessary to follow all these conditions when establishing a new calibration curve.

Spectrophotometric Method

Apparatus.—The following apparatus was utilized in this work: Pyrex 50-ml. centrifuge tubes; Pyrex emulsion tubes, 50 ml. graduated in ml., 25 x 200 mm.; Pyrex chromatographic adsorption tube, 6 x 80 mm., with 30-ml. reservoir above and a 1 x 15 mm. capillary constriction below (see Fig. 3); Pyrex Kramer-Gittleman tubes; and an air pressure system with a gauze filter and mercury manometer (see Fig. 3).

In addition to the above, a Beckman Model DU spectrophotometer, an International clinical model centrifuge, glass rods, No. 6½ one-hole rubber stoppers, a hot plate, 4-mm. glass beads, a balance, and a hot water bath were used.

Reagents.—The reagents used were as follows: ethanol, 96%; chromatographic adsorption alumina, 80–200 mm., Eimer and Amend; pure silica sand; distilled or deionized water; and concentrated nitric acid.

Method.—Oxalated or heparinized blood (2 ml.) is added to 20 ml. of alcohol in a centrifuge tube, and

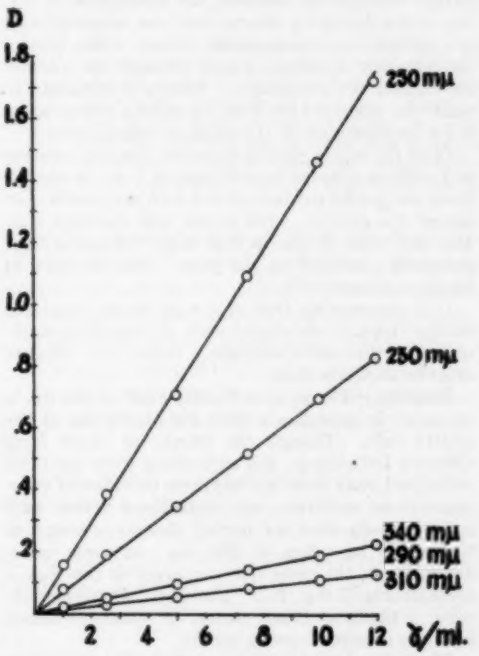

Fig. 2.—Calibration curves. Khellin in alcoholic extracts.

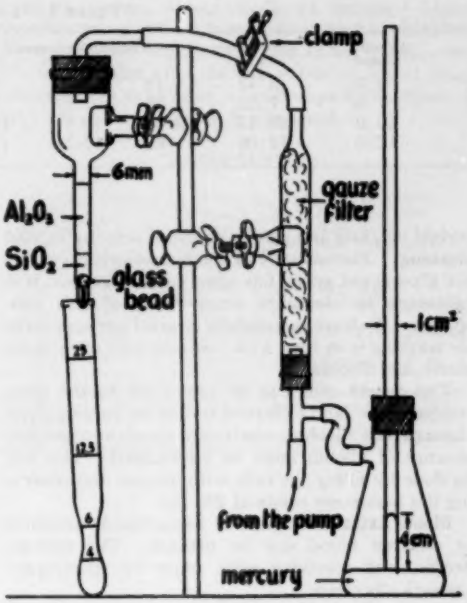

Fig. 3.—Apparatus.

the mixture is stirred with a glass rod until fine clumps appear; it is then centrifuged at 2,500 r. p. m. for five minutes. The supernatant liquid is decanted into the graduated Pyrex tube and evaporated over a boiling water bath.

The precipitate is washed by stirring with the same glass rod after addition of 5 ml. of alcohol and is centrifuged again for five minutes; the wash is decanted into the tube containing the alcoholic extract and evaporation is continued until a volume of 7 ml. is reached.

The concentrated alcoholic extract is cooled to room temperature and is then passed through a 6-mm. washed column, previously packed with 0.5 Gm. of pure silica sand at the bottom and 1.0 Gm. of alumina, under a pressure of approximately 40 mm. of mercury. The total filtrate is collected in Kramer-Gittleman tubes. The column is washed with alcohol until the eluate reaches the 12.5-ml. mark, and the combined filtrate and washings are then mixed by inverting two or three times.

The optical density is read at 250 mμ, using 96% alcohol as a blank; from this reading is subtracted that of a blank prepared from the same blood free from khellin. The amount of khellin may then be calculated in μg./ml. of alcoholic extract by comparison with standards. This value can be reported in mg./L. of blood by using 6.25 as factor. If a blank of the same blood being examined cannot be obtained, the value of 0.300 may be subtracted from the optical density reading. This value is the range of 10 normal blood samples free from khellin, treated identically according to this method, and is equivalent to 2 μg./ml. of alcoholic extract.

Discussion.—The apparatus must be scrupulously clean from a spectrophotometric point of view. Until cleaned, every piece of soft glass apparatus which contained khellin must be considered heavily contaminated due to the fact that khellin is ad-

TABLE I.—KHELLIN, μG./ML.

Amount Added	Amount Recovered					Mean Values	Mean % Recovery
	1	2	3	4	5		
5.0	5.42	4.19	5.26	5.02	4.51	4.9	98.00 ± 14.20
7.5	7.52	7.36	7.68	7.50	7.44	7.5	100.00 ± 2.40
10.0	10.13	9.80	10.00	10.19	9.88	10.0	100.00 ± 2.00
12.0	12.06	11.69	11.88	11.83	12.04	11.9	99.17 ± 1.75

sorbed on glass and can be removed only by careful cleaning. The amount of khellin adsorbed on Pyrex glass is not great, but when blanks are used it is necessary to eliminate every trace of this substance. We have successfully cleaned our apparatus by washing it in turn with concentrated nitric acid, water, and alcohol.

The quartz cells can be submitted to the same treatment or can be cleaned simply by passing them through hot alcohol. In every case the complete absence of khellin must be ascertained. This can be done by filling the cells with alcohol and observing if a maximum exists at 250 mμ.

Blood Extraction.—Either heparinized, oxalated, or citrated blood can be utilized. The syringe, needle, and receiving tube must be spectrophotometrically clean.

Khellin Extraction.—We are entirely in accord with the statement of Anrep, Barsoum, and Kenawy (7) that the ratio 1:10 established in their first extraction is the most satisfactory. Nevertheless, we consider their subsequent extraction with water and chloroform unnecessary. Chloroform generally contains impurities which interfere with the ultraviolet absorption spectra. Notwithstanding this, we have used the complete method of extraction and have submitted the extracts to chromatographic adsorption; satisfactory results have been obtained. However, the method is very cumbersome and the results are most satisfactory when the use of chloroform is omitted. Together with khellin, great quantities of chromogens are extracted by alcohol. Chromatography eliminates them and renders solvent extractions unnecessary.

Shaking and Centrifuging.—Diffusion of khellin in alcohol is relatively slow. We attempted to obviate this difficulty first by allowing the alcohol to stand together with the blood for twenty minutes, stirring the mixture with a glass rod from time to time. This made the method considerably longer. On adding blood to alcohol instead of using the reverse order, the blood forms sufficiently fine clumps to make the diffusion complete after stirring for one minute. The time of centrifuging does not modify the final results significantly. We have centrifuged for two to three minutes at 2,500 r. p. m., although a better decantation is obtained after 5 minutes.

Concentration of the Extracts.—As the final volume must be less than 12.5 ml. and the amount of alcohol used in the extraction is more than 20 ml., it is necessary to reduce the volume of the extract by evaporation. It could be assumed that by reducing the volume of the extract to 2 or 3 ml., the final volume, after chromatography, could be brought to 4–6 ml. in the Kramer-Gittleman tubes, increasing in this manner the sensitivity to amounts smaller than 1 μg./ml. This is not possible, however, due to the fact that the substances carried over to the alcohol together with the khellin render the extracts turbid if the volume is smaller than 6 ml.

Chromatography (10, 11).—Before passing the extracts through the alumina column, they were cooled to room temperature. The passage through the adsorption column must be at a constant pressure controlled with the aid of a mercury manometer and a clamp as illustrated in Fig. 3. Due to the fact that the alumina is partially carried along by alcohol, it was found necessary also to use a column of silica. To standardize the adsorption of the blanks, it was necessary to determine the weight of both silica and alumina.

The use of such small amounts of adsorbent is justified due to the small quantities of the substances to be adsorbed. Khellin is adsorbed weakly in the alumina column, permitting an easy elution. Conversely, the chromogens are, in general, strongly adsorbed at the top of the column which enables them to be separated. This eliminates interference in the region of 250 mμ.

In order to determine the alcohol-soluble impurities and blank from the alumina, several 4-ml. portions of alcohol were passed through the column and their absorption was read in the region of 220–350 mμ. After the first 4 ml. of alcohol has been passed through the column, the absorption at 250 mμ. of the following eluates does not interfere with the method to a measurable extent. The greater the quantity of solvent passed through the column, the smaller the absorption. Thus it is necessary to wash the column with 5 ml. of alcohol before using it for fractionation of the alcoholic blood extract.

Once the whole extract is passed, the total elution of khellin is effected by addition of 6 ml. of alcohol from the graduated tube which had previously contained the extract. The eluate will therefore contain any trace of khellin that might otherwise have remained adsorbed on the glass. The recovery of khellin is about 100%.

It is noteworthy that traces of bloom from the rubber stoppers are eluted with the khellin and absorb the ultraviolet strongly. Hence the stoppers must be perfectly clean.

Reading.—The optical density read at 250 mμ is corrected in accordance with the absorption of the quartz cells. Though the blanks of blood from different individuals, and even those from the same individual, may vary in absorption on different occasions, these variations are maintained within such narrow limits that an optical density average of 0.300 may be taken at 250 mμ. In some cases, nevertheless, the error that can occur in this way is considerable (5 mg./L.). Therefore, whenever possible, a blank of blood should be obtained before injecting or administering khellin.

When the aim is merely to continue the increase or decrease of the khellin level in the blood, the average of optical density can be successfully employed.

Errors.—The errors inherent in this method are numerous, though of small magnitude. The extraction is not complete but it can be so regarded considering that it is better than 95%.

Results shown in Table I were determined by adding measured amounts of khellin to khellin-free blood, submitting the blood standards to the process explained, and measuring the concentration in the eluates.

Other sources of error are the transfer of the extracts to the absorption column, the chromatographic adsorption procedure, the spectrophotometric readings, and, finally, the difference in absorption which can exist between the blood sample extracted as a blank and the blood itself from the same patient after khellin administration. If the best working conditions are maintained, all these errors will not exceed 1 mg./L. in the final reading. The direct reading of alcoholic solutions of khellin has a much greater sensitivity and yields values of 0–15 μg./ml. with an error of 0.1 μg.

In the course of our work we analyzed blood samples under different conditions and have found no appreciable interference by other substances. We have found reproducible values in blood from blood blanks even after several days if the blood is kept refrigerated and well stoppered.

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Laxatives: Chemical Structure and Potency of Phthaleins and Hydroxyanthraquinones*

By MAX H. HUBACHER, SIDNEY DOERNBERG, and ARTHUR HORNER†

A review is presented of laxatives of organic structure, mostly phthaleins, phthalein derivatives, and hydroxyanthraquinones. A critical evaluation is made of previous theories concerning the relationship between laxative potency and chemical structure and of the methods of assaying. Thirty-six organic compounds were prepared and tested for laxative action, using the rhesus monkey as a test animal. A precise rule for correlating potency and molecular structure cannot be stated at this time. However, it will be seen that compounds which have laxative properties possess at least two phenolic hydroxyls and at least three rings in their molecules.

SEVERAL hypotheses have been advanced in the attempt to correlate laxative action of organic compounds with their chemical structure. Most of these postulates obviously are untenable and have often served as the source of new errors. The principal cause for this inaccuracy lies in the fact that reliable methods for the determination of laxative properties have not been used in most cases.

A great variety of test animals were used in the determination of cathartic action of drugs, namely crustaceans (1), fish (2), frogs (3), mice (2–14), rats (3, 5, 15–18), guinea pigs (17, 19–21), rabbits (2, 3, 19, 22), cats (2, 16, 22, 23), dogs (2, 5, 16, 22, 24–26) and monkeys (7, 16, 27–29).

Williams, Abramowitz, and Killian (16) were the first to demonstrate clearly the value of the rhesus monkey as a test animal in the study of the laxative activity of phenolphthalein. The suitability of the monkey was fully confirmed by others (7, 27–29), who also showed that this animal could be used in the evaluation of other laxatives, its response being analogous to that in man. The monkey is the most reliable animal for screening substances for their value as laxatives.

Mice seem to be suitable for testing the potency of such cathartics as senna and rhubarb, i. e., drugs in which glucosides of hydroxyanthraquinones probably are the active constituents. Considerable literature exists on this assay method (4, 8–14). The action of these hyroxyanthraquinones is similar in man and in

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† The authors are indebted to Dr. A. A. White, Jr., for editorial assistance.

mice, but their sensitivity is only about $^1/_{100}$ of that in man (4, 7, 10).

Much of the data on laxative potency of compounds found in the literature is confusing and of little comparative value because the experiments were performed on animals whose response to laxatives in most cases was different from that of man. To cite but two examples, phenolphthalein is an effective laxative in man and monkey (7, 16, 27–29) but is inactive in mice (3, 7), rats (3, 16, 18), guinea pigs (19), rabbits (3, 19, 22), cats (16, 22), and dogs (16, 22), while 3-(*p*-hydroxyphenyl)isocoumarin, which is reported to be very active in guinea pigs (20) and purgative in cats (23), was found by us to be inactive in man and monkey.

The excised intestine has been used for testing drugs for laxative potency (30, 31). This method, however, is of questionable value because it does not take into account the changes that might take place in the ingested drug before reaching the site of action (17, 23). Neither does this method reveal what secretory mechanism is stimulated that contributes to the action of a laxative. Furthermore, it is not at all specific for laxatives, as numerous other drugs cause spasmodic contractions of the excised intestine, yet show no laxative effect in the living animal or man.

The action of laxatives such as phenolphthalein in man and monkey, as contrasted to their inertness in other animals, suggests a difference in physiology of the intestines. Vamossy, the discoverer of the cathartic action of phenolphthalein, writes that neither he nor anybody else has been able to discover the reason for this difference (32, 33).

Ogata and Kondo (34) proposed the hypothesis that the diphenylmethylene group, $C_6H_5—\underset{|}{\overset{|}{C}}—C_6H_5$, is responsible for the cathartic action of a series of compounds, and that the carbonyl group, >C=O, is of minor importance. They determined the potencies by the excised intestine method; their results, therefore, are subject to the shortcomings of this method. These investigators also overlooked the fact that the majority of the phthaleins are inactive as laxatives, yet possess the two groups in question in their molecules.

According to Silberschmidt (26), the presence of free phenolic groups is essential for cathartic action, while the presence of a —CO—NH— group seems to be the cause of the increased action. He bases his conclusions on four compounds. A substance which fulfills his postulate for a laxative is phenolphthaleinimide, yet this compound (No. 19) exhibits practically no cathartic action.

Kaufmann (23) tested a great number of aromatic compounds, using the cat as a test animal, and advanced a hypothesis as to what chemical grouping is necessary for laxative activity. He concludes that the substituted 4,4′-dihydroxydiphenylmethylene group, $(p)HO—\underset{|}{\overset{|}{C_6H_5—C—C_6H_3OH(p)}}$, is responsible for cathartic action, and he therefore calls this group the "laxative group." There are indeed many compounds which have this grouping in their molecule (see Table II) and are good laxatives. On the other hand, there are numerous compounds which embody Kaufmann's "laxative group," but are inert, or practically so, as laxatives. Examples of such inactive compounds are 4,4′-dihydroxybenzophenone, phenolphthalin, phenolphthaleinimide, and phenol-1,2-naphthalein. A compound which Kaufmann first prepared and named phenolhomophthalein was used by him in support of his theory. Subsequently, Buu-Hoï (35) showed that this compound is not a phthalein but an isocoumarin derivative devoid of Kaufmann's "laxative group" (No. 28). Kaufmann's hypothesis has appealed to many workers in this field and is often quoted in textbooks.

Easson and co-workers (5) used Kaufmann's hypothesis and prepared several new compounds having the 4,4′-dihydroxydiphenylmethylene group. Their findings have little value, primarily because the potencies were determined on mice, rats, and dogs. Furthermore, the structure of some of their compounds has not been adequately established and only scant experimental data are given.

Buu-Hoï (20) attributes the laxative action of certain organic compounds to the presence of a lactone group, assigning only secondary role to the phenolic hydroxyls. He calls the first group the "toxophoric" and the hydroxyl the "haptophoric" group. Again, this theorizing falls short of acceptability because Buu-Hoï bases his observations on only four compounds, while many others embodying these requisites exhibit no laxative activity.

Loewe and Hubacher (36) determined the potencies of 22 compounds belonging to the phthalein, hydroxyanthraquinone, and the indolinone classes. Many of these compounds are included in our series of experiments. It was concluded, with some reservations, that one carbonyl group, >C=O, and two phenolic hydroxyls in *para* position are essential for imparting laxa-

tive properties to a compound. However, many exceptions to these rules can be mentioned, e. g., thymolphthalein, phenolphthaleinimide, and others, all of which exhibit little activity yet have the requisite groupings.

Loewe, in a more recent publication (29), studied the laxative properties of a large number of derivatives of 3,3-diphenylphthalide. He expresses his findings in a number of rules. However, their complicated nature and the numerous exceptions detract somewhat from their value. Benzene-resorcinolphthalein (No. 5), for example, has high laxative potency, yet according to Loewe's rules it should not have any laxative properties.

Ziegler and his co-workers (37) found that the molecule of some laxatives is split into two parts when made to react under specified conditions with diazonium compounds. They suggest that a correlation exists between cathartic properties and the tendency to cleavage. They showed that phenolphthalein, *o*-cresolphthalein, and diphenolisatin react with *p*-nitrodiazonium chloride. However, since *o*-cresolphthalein has no laxative properties, this reasoning appears questionable.

EXPERIMENTAL[1]

Preparation of the Compounds

The methods of preparation and purification of the 36 known compounds discussed in this paper are shown in Table I. The compounds possessing laxative potency are shown in Table II. In six cases, where the method of preparation has been improved over that shown in the literature, the procedure is described below in some detail. The preparation and structure proof of phenol-*o*-cresolphthalein is given because the compound obtained in this work has a melting point 97° higher than the one ascribed to it by Ghatak (38).

Benzene-catecholphthalein or 3-Phenyl-3-(*m,p*-dihydroxyphenyl)phthalide (No. 6).—A quantity of 300 ml. of benzene and 22.0 Gm. of catechol was added to the acid chloride, prepared in the usual manner from 45.2 Gm. of *o*-benzoylbenzoic acid (m. p. 128°) and 36 Gm. of thionyl chloride. The mixture, which gives off HCl, was heated from 21 to 50° over a period of three hours. After standing overnight, the crystals were filtered off and washed with benzene; yield, 53–56 Gm., m. p. 167–169°. By recrystallizations from acetic acid, colorless crystals melting at 168.3 to 169.5° were obtained. Its diacetyl derivative melted at 145–147°.

The melting points recorded in the literature for this phthalein are 160–161° (39), 86° (40), and 170–171° (41).

Phenol-*o*-cresolphthalein or 3-(*p*-Hydroxyphenyl)-3-(*p*-hydroxy-*m*-tolyl)phthalide (No. 10).—A mixture of 21.6 Gm. of *o*-cresol,[2] 24.2 Gm. of 2-(*p*-hydroxybenzoyl)benzoic acid (42), 10 Gm. of anhydrous zinc chloride, and two drops of conc. sulfuric acid was stirred for three hours at 120°. Dilute hydrochloric acid was added and the unreacted *o*-cresol was steamed out. The crude phthalein (34–36 Gm., m. p. 200–207°) was recrystallized from acetic acid (1 Gm. in 5 ml.) or from 41% ethanol (1 Gm. in 12 ml.). The total yield of recrystallized phenol-*o*-cresolphthalein was 26.1 to 28.9 Gm. (76–86%), m. p. 208–210°.

This same phthalein was also obtained by stirring (for five hours at 120°) a mixture of 14.1 Gm. of phenol, 12.8 Gm. of 2-(*p*-hydroxy-*m*-tolyl)benzoic acid (43), 5 Gm. of anhydrous zinc chloride, and one drop of conc. sulfuric acid.

The pure phenol-*o*-cresolphthalein melted at 213.3 to 214.9° [reported, 117° (38) and 211–213° (29)] and dissolved in dilute alkalies, producing a red color.

Anal.—Calcd. for $C_{21}H_{16}O_4$: C, 75.94; H, 4.84; mol. wt. 332. Found: C, 75.56; H, 4.53; mol. wt. 328 [Signer method (44)].

The structure of this phthalein is evident from its two syntheses, as well as from the fact that we succeeded in splitting its oxime by the Friedlaender method (43) into 2-(*p*-hydroxybenzoyl)benzoic acid and an aminophenol melting at 167–172°. The latter was found to be identical with a sample of 4-amino-2-methylphenol (45).

Phenol-1,8-naphthalein or 3,3-Bis(*p*-hydroxyphenyl)-[1,8]naphthalide (No. 24).—A mixture of 19.8 Gm. of naphthalic anhydride (m. p. 269–272°), 37.6 Gm. of phenol,[2] 100 ml. of *s*-tetrachloroethane, and 60 Gm. of anhydrous $AlCl_3$ was stirred for three days at 105–110°. First the solvent was steamed out, then the dark, semisolid mass was dissolved in 200 ml. of alcohol, leaving unreacted naphthalic anhydride undissolved. The filtrate was evaporated and the residual mass dissolved in 1 L. of normal sodium hydroxide. Additional naphthalic anhydride was removed and the red filtrate was saturated with CO_2. This precipitated the phthalein, leaving the 8-(*p*-hydroxybenzoyl)-1-naphthalic acid in solution.

The crude phthalein was dissolved in 1.5 L. of hot acetic acid; after standing for several days at 15°, colorless crystals formed (6.4 to 10.0 Gm.; 17–27% yield). The pure phenol-1,8-naphthalein melted at 267.9 to 270.0°. Its diacetyl derivative melted at 203.0 to 203.5°.

The melting points reported for this phthalein are 200° (46) and 265–266° (47).

3-(*p*-Hydroxyphenyl)isocoumarin (No. 28).—A mixture of 32.4 Gm. of homophthalic anhydride (48), 18.8 Gm. of phenol, and 20 Gm. of $SnCl_4$ was stirred for one hour at 100–105°. The solid, colored reaction mass was treated with hot dilute hydrochloric acid. The isocoumarin derivative remained undissolved while the unreacted homophthalic acid (14–16 Gm.) dissolved and then crystallized from the filtrate. The 3-(*p*-hydroxyphenyl)isocoumarin (22–24 Gm.) was crystallized from 300 ml. of acetic acid, yielding 17 Gm. of crystals (m. p. 227–228°). The pure compound melted at 230.1 to 231.5° [reported, 227° (35)].

Emodin or 1,3,8-Trihydroxy-6-methylanthraquinone (No. 35).—A mixture of powdered buckthorn bark and 1.0 *N* hydrochloric acid was kept for sev-

[1] All melting points are corrected except where noted.

[2] This is a 100% excess over theory, the excess acting as a solvent for the reaction.

[2] This is a 100% excess over theory.

TABLE I.—PREPARATION OF COMPOUNDS AND THEIR LAXATIVE POTENCIES

No.	Name	Preparation	Purification	M.P., ° C.	Laxative Potency
	3,3-Diphenylphthalide and Hydroxy Derivatives				
1	3,3-Diphenylphthalide	(64)	Distillation *in vacuo*, then crystallized from ethanol	115.6–116.1	0.1
2	3-Phenyl-3-(*p*-hydroxyphenyl phthalide	(65, 66)	Crystallizations from acetic acid	170.1–170.4	0.3
3	Phenolphthalein	U. S. P. phenolphthalein was used	.....	261.3–262.1	1.0
4	Isophenolphthalein or 3-(*o*-hydroxyphenyl)-3-(*p*-hydroxyphenyl)phthalide	(67)	Crystallizations from acetic acid	205.5–206.1[a]	Inactive
5	Benzene-resorcinolphthalein or 3-phenyl-3-(*o,p*-dihydroxyphenyl)phthalide	(39)	Crystallizations from chloroform and the crystals dried at 120°	198.2–200.0[b]	2.1
6	Benzene-catecholphthalein or 3-phenyl-3-(*m,p*-dihydroxyphenyl)phthalide	Described in text	.....	168.3–169.5	0.4
	Esters and Ethers of Phenolphthalein				
7	Diacetylphenolphthalein	By acetylation of phenolphthalein	Crystallizations from alcohol	146.5–147.3	0.4
8	Monomethyl ether of phenolphthalein	From 2-(*p*-methoxybenzoyl)-benzoyl chloride and phenol in benzene solution (65)	Crystallized from acetic acid	140.5–142.2	Inactive
9	Dimethyl ether of phenolphthalein	From phenolphthalein and dimethylsulfate in 5 *N* sodium hydroxide	Crystallizations from alcohol	100.5–1001.1	0.1
	Alkyl- and Aryl-substituted Phenolphthalein				
10	Phenol-*o*-cresolphthalein or 3-(*p*-hydroxyphenyl)-3-(*p*-hydroxy-*m*-tolyl)-phthalide	Described in text	.....	213.3–214.9	0.1
11	*o*-Cresolphthalein	From *o*-cresol, phthalide anhydride, and anhydrous zinc chloride at 120°	Crystallizations from alcohol	221.3–223.6	Inactive
12	Thymolphthalein	(69)	Crystallizations from acetic acid	252.4–253.1	Inactive
13	Carvacrolphthalein	(69)	Crystallizations from acetic acid	293.5–294.7	Inactive
14	3,3-Bis(*m*-phenyl-*p*-hydroxyphenyl)phthalide	(70)	Crystallizations from 41% alcohol	244.2–244.8[c]	Inactive
	Compounds Having the Group (*p*)HO—C_6H_4—C—C_6H_4—OH(*p*) in Common				
15	4,4′-Dihydroxybenzophenone	(72)	Crystallizations from water (1 Gm. in 120 ml.)	216.6–217.1	0.1
16	Phenolphthalin or 4′,4″-dihydroxytriphenylmethane-2-carboxylic acid	By reducing phenolphthalein in 5 *N* sodium hydroxide with zinc dust	Crystallizations from 41% alcohol	237.6–238.5	Inactive
17	Phenolphthalol or 2-(4′,4″-dihydroxybenzhydryl)-benzyl alcohol	(73)	Crystallizations from water (1 Gm. in 750 ml.)	201.5–201.9	2.9
18	4,4′-Dihydroxytriphenylmethane	By decarboxylation of phenolphthalin (74)	Crystallizations from 41% alcohol	164.3–165.5	0.9
19	Phenolphthaleinimide	From 3.2 Gm. of phenolphthalein in 30 ml. strong ammonia for 3 days at 25°	.....	261.6–264.2	0.1
20	Diphenolisatin or 3,3-bis(*p*-hydroxyphenyl)oxindole	(58)	Crystallizations from 41% alcohol	262.2–265.7	41.7
21	Diacetylderivative of above also called Isacen	By acetylation of No. 20 with acetic anhydride	Crystallizations from 41% alcohol	239.6–240.2[d]	20.9
22	Phenol-1,2-naphthalein	(75)	Crystallizations from acetic acid	268.1–270.2	Inactive

(*Continued on page 27*)

TABLE I (*Continued from page 26*)

No.	Name	Preparation	Purification	M.P., ° C.	Laxative Potency
23	Phenol-2,1-naphthalein	(75)	Crystallizations from acetic acid	291.1–292.4	0.5
24	Phenol-1,8-naphthalein	Described in text	Crystallizations from acetic acid	267.9–270.0	5.5
	Phenolphthalein Having the OH Group Replaced by Others				
25	Anilinephthalein	(76)	Crystallizations from methanol	202.5–203.1	Inactive
26	3,3-Di-*p*-tolylphthalide	(77)	Crystallizations from alcohol	115.6–117.0	Inactive
	Miscellaneous Compounds				
27	Phenolphthalidein or *o*-di-(*p*-hydroxybenzoyl)-benzene	(78)	Crystallizations from 41% alcohol	228.0–228.8	Inactive
28	3-(*p*-Hydroxyphenyl)isocoumarin	Described in text	Crystallizations from acetic acid	230.1–231.5	Inactive
29	Fluoran	Obtained as a by-product in the manufacture of phenolphthalein	Sublimation at 150° and high vacuum (49), then crystallizations from alcohol	183.9–184.2	Inactive
	Hydroxyanthraquinones				
30	1-Hydroxyanthraquinone	From 2-(*o*-hydroxybenzoyl)benzoic acid in a yield of 29% (78)	Sublimation in high vacuum (49)	193.4–194.2	Inactive
31	2-Hydroxyanthraquinone	By diazotation of 2-aminoanthraquinone in conc. sulfuric acid, etc. (79)	By sublimation in high vacuum (49)	310.5–311.4	Inactive
32	Quinizarin or 1,4-dihydroxyanthraquinone		By sublimation in high vacuum (49)	200.4–200.9	0.2
33	Chrysazin or 1,8-dihydroxyanthraquinone	Available commercially under the name of Danthron (Winthrop Chem. Co.)	By sublimation in high vacuum (49)	193.6–194.0[e]	0.4
34	Chrysophanol or 1,8-dihydroxy-3-methylanthraquinone	Isolated from chrysarobin U. S. P., by the procedure of Gardner (80)	By sublimation at 150° and 10 μ pressure (49)	195.0–195.6[f]	0.2
35	Emodin or 1,3,8-trihydroxy-6-methylanthraquinone	Described in text	Sublimation at 150° and 10 μ pressure (49)	255.2–260.0	0.2
36	Anthrapurpurin or 1,2,7-trihydroxyanthraquinone	Described in text	.....	352–355 (uncorr.)	0.2

[a] Its diacetyl derivative melted at 168.2–168.6°.
[b] Its diacetyl derivative melted at 140.9–141.9° [reported, 137° (68)].
[c] The literature records a melting point of 234–235° (70) and 242° (71).
[d] The melting point of 242° found in the literature (59) is probably too high.
[e] This compound was found to be identical with the German laxative Istizin, m. p. 193.0–194.0°, of the Friedrich Baeyer Company.
[f] Was found to be identical with a sample of synthetic chrysophanal kindly supplied by Dr. H. Mühlemann (10).

eral hours at 70°. The filter cake was extracted with 41% ethanol. The combined dark brown filtrate was then extracted with many portions of benzene. The combined yellow benzene layer was in turn extracted with 1.0 *N* sodium hydroxide and the united alkaline, deep purplish-red extract was saturated with CO_2. The crude emodin was purified by crystallizations from alcohol and by fractional sublimation at 180° and 10 μ pressure (49). A small quantity of a by-product, more volatile and lower-melting, also was separated. The pure emodin melted at 255.2 to 260.0° and was found to be identical with a sample of synthetic emodin kindly sent by Dr. H. Mühlemann (10).

Anthrapurpurin or 1,2,7-Trihydroxyanthraquinone (No. 36).—This compound was isolated from "Alizarin Red B Paste."[g] The dried paste (5.0 Gm.) was extracted in a Soxhlet apparatus with 100 ml. of alcohol. The crystals were then fractionally sublimed (49). The sublimate (0.8 Gm.) obtained at 190° and 10 μ pressure melted at 295° and proved to be alizarin. The sublimate (1.82 Gm.) obtained at 250° and 10 μ pressure formed crystals of strong orange color and melted at 352–355° (uncorr.); it is the anthrapurpurin. Its triacetyl derivative melted at 231.0 to 233.4°.

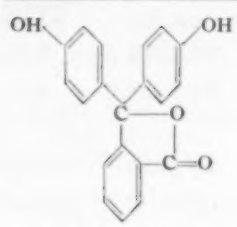
[g] National Aniline and Dyestuffs Corp., New York 6, N. Y.

Determination of the Laxative Potency

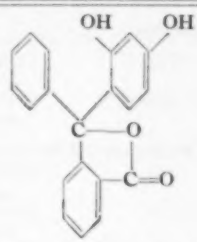
All compounds were ground to the smallest particle size, previous to administration. Water-insoluble laxatives of different particle size gave varying results; the smaller particle size exhibits a slightly higher potency.

The laxative potency (LP) was determined on the rhesus monkey by the previously described method (28). This is based on the equieffective

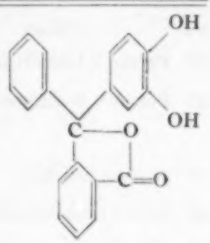
TABLE II.—STRUCTURAL FORMULAS AND LAXATIVE POTENCIES (LP)



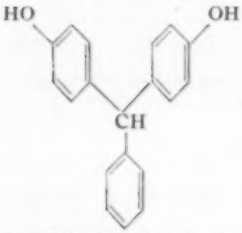
Phenolphthalein
LP = 1.0



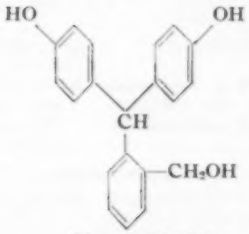
Benzene-resorcinolphthalein
LP = 2.1



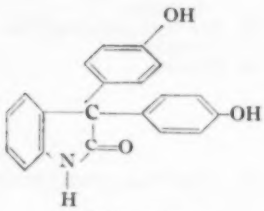
Benzene-catecholphthalein
LP = 0.4



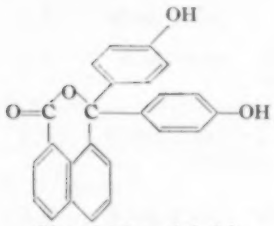
4,4′-Dihydroxytriphenylmethane
LP = 0.9



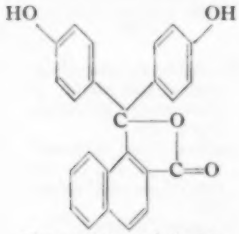
Phenolphthalol
LP = 2.9



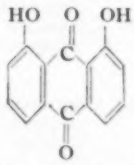
Diphenolisatin
LP = 41.7



Phenol-1,8-naphthalein
LP = 5.5



Phenol-2,1-naphthalein
LP = 0.5



1,8-Dihydroxy-anthraquinone
LP = 0.4

dose principle, i. e., by determining the minimum effective or threshold dose and comparing it with a standard. Phenolphthalein U. S. P. was chosen as a standard and given the value of 1.0. Phenolphthalein fulfills the requirements for a biological standard very well because of its purity, its stability, and its unvarying biological activity.

Numerous investigators have resorted to forced feeding of the animals. Based on our experience, this method has proved unsuitable when testing substances for cathartic action. The animals thus fed become emotionally disturbed, producing loose stools even with the administration of a placebo.

We have found our method to yield consistently concordant values comparable with those obtained in humans. Several compounds, namely Nos. 3, 4, 5, 12, 24, and 25, have also been tested in man and were found to give similar responses.

The average dose for the rhesus monkey, on a mg./Kg. basis, is about four times higher than for man.

The average human laxative dose (LD) of any of the compounds can be calculated from the laxative potency factor (LP) by the following formula

$$L\,D = \frac{0.12}{LP}\ Gm.$$

in which 0.12 is the average human dose in grams for phenolphthalein U.S.P.

The results of our laxative potency determinations are shown in Table I. Our values confirm those obtained on some of the compounds assayed by Loewe and Hubacher (36). The laxative potency figures (LP) are in proportionate relationship because all of the bioassays were made under standard conditions on one type of animal, the rhesus monkey.

If doses 10 times the Minimum Effective Dose of phenolphthalein U. S. P. did not produce any laxative effect, we termed that compound inactive as a laxative. While it is true that ingestion of larger doses might produce laxation, for practical purposes such compounds can be considered inactive.

DISCUSSION

We have prepared and tested 36 known compounds, comprising phthaleins, phthalein derivatives, and hydroxyanthraquinones. Only carefully purified compounds were used in our pharmacological tests because instances are known where the crude compound has shown a higher potency than when purified (28). The laxative potency of these compounds is shown in Table I.

The postulates advanced by certain investigators in this field have some validity but are vitiated by their limited applicability. For each example offered to prove the theory, there are an equal or even greater number of exceptions. Kaufmann's hypothesis (23) appears to have the greatest acceptance, but even its application cannot be generalized.

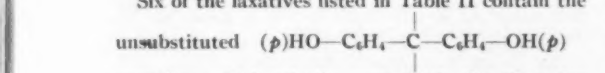
At one time, we assumed that there was a relationship between the color fading of phthaleins in alkaline medium and their laxative potency. This permanent fading is caused by air oxidation (50), but this hypothesis had to be discarded when it was discovered that phenol-1,8-naphthalein, which has a high laxative potency, does not fade at all.

At the present time, we cannot offer a precise rule whereby the chemical structure and the laxative potency of a compound may be correlated. We note, however, that compounds having laxative activity, as listed in Table II, have one characteristic in common, namely they possess two phenolic hydroxyls. They contain at least three rings in their molecule; yet, not all such compounds are laxatives.

These hydroxyls seem to be of some significance in producing laxation. When one or all hydroxyls are removed from a compound with cathartic properties, the resulting compound shows reduced or no laxative action. Thus, compounds Nos. 1, 2, 30, and 31 are inactive, while the corresponding di- and trihydroxy compounds 3, 5, 6, 32, 33, 34, 35, and 36 are active. When the two OH groups in phenolphthalein are replaced by amino groups, the resulting aniline-phthalein (No. 25) is inactive.

We can confirm two observations made by Loewe (29), namely, that when one or both OH groups are esterified, and especially when etherified, as is the case with compounds Nos. 7, 8, and 9, the laxative properties of the compounds are reduced or eliminated. Introduction of alkyl or aryl groups into these rings of phenolphthalein, which contain the OH groups, also has the same reducing effect as in compounds Nos. 10, 11, 12, 13, and 14.

Six of the laxatives listed in Table II contain the



unsubstituted $(p)HO—C_6H_4—\underset{\displaystyle |}{\overset{\displaystyle |}{C}}—C_6H_4—OH(p)$

grouping which Kaufmann called the "laxative group." It should not be construed as has already been pointed out, that any compound containing this group in its molecule is a cathartic. Thus, compounds Nos. 15, 16, 19, and 22 have this group, yet show little or no laxative properties.

These studies also corroborate the observations made in other fields, where structure-activity relationships have been studied, that even a minor change in the molecule of a pharmacologically active compound makes it inactive.

The structural formulas of the 9 most potent laxatives found among the 36 compounds studied by us are shown in Table II. They all happen to be practically water-insoluble. They are:

Phenolphthalein.—The discovery by Vamossy (51) in 1900 that this compound possessed laxative activity made available a stable, tasteless, nonirritant laxative, the value of which has not diminished during the intervening 52 years (32, 52).

According to newer investigations, phenolphthalein acts by its stimulating influence primarily on the musculature of the large intestine (53) and in no way affects the kidney (54) or liver (55).

The dose recommended in the U. S. P. XIV is 1 gr. The studies of McGuigan, Steigmann, and Dyniewicz (56), confirmed by us, indicate that this dose is inadequate, 2 gr. being a more satisfactory average dose for adults. The British Pharmacopoeia gives the dose as 1 to 5 gr.

The former belief that allergic skin eruptions caused by phenolphthalein are of frequent occurrence has been disproved (52). The low toxic index of phenolphthalein is evidenced by the fact that in children, accidental overdoses as high as 130 gr. produced only laxation, without harmful effects (57).

A grade of phenolphthalein which is known in the trade as yellow phenolphthalein is 2.5 times more potent than the U. S. P. grade of phenolphthalein (28).

Benzene - resorcinolphthalein.—This phthalein, a position isomer of phenolphthalein, was found to be twice as active as the latter.

Benzene-catecholphthalein.—This phthalein likewise is an isomer of phenolphthalein.

4,4′-Dihydroxytriphenylmethane.—This compound, a part of the phenolphthalein molecule which can be prepared from the latter, has a potency approximating that of phenolphthalein.

Phenolphthalol.—This tasteless and odorless compound is not a phthalein but a phenolic alcohol. It was found to be almost three times as active as phenolphthalein.

Diphenolisatin.—This compound was first prepared by Baeyer (58); 40 years later, in 1925, Guggenheim discovered its laxative properties (59). Its diacetyl derivative is marketed under the trade name of Isacen.[5] Diphenolisatin is one of the most potent organic laxatives known, being 41.7 times stronger than phenolphthalein.

This class of compounds, heretofore, has not been found in nature. Very recently, Baum, Sanders, and Straub (60) announced that "diphenylisatin has been identified in the chloroform fraction of an extract of California prunes." They used canned prune juice as their starting material. Their identification of this compound is based solely on the similarity of the absorption spectra curves (not reproduced in their paper) with an analogous compound. The melting point of the isolated compound is not given, nor any estimation of the amount present in unprocessed prunes.

Phenol-1,8-naphthalein.—Kaufmann reported this phthalein to be laxative in cats (23) and Loewe (29) in monkeys. We found it to be five and one-half times as active as phenolphthalein in the monkey. Its isomer, the phenol-2,1-naphthalein, has a potency of only 0.5, and phenol-1,2-naphthalein is inactive.

Hydroxyanthraquinones.—As a result of the discovery of the presence of hydroxyanthraquinones in some botanical laxatives, these compounds, either isolated from plant material or made synthetically, became the subject of many investigations regarding their purgative action. Those included in our series of experiments are only a few of this group of compounds, as their isolation, in most cases, is a time-consuming undertaking.

The pure hydroxyanthraquinones are all yellow to orange colored compounds, imparting the color to urine when ingested. They are only mildly laxa-

[5] Hoffman-LaRoche, Inc.

tive. The highest potency was found in 1,8-dihydroxy anthraquinone, a compound still used as a laxative in Germany under the trade name of Istizin (61). The 1,2,7-trihydroxyanthraquinone was marketed at one time in the form of its diacetyl derivative under the trade name of Purgatin (62), with a recommended dose for adults of 0.5 Gm.

The pharmacology of the hydroxyanthraquinones has recently been discussed by Schultz (63).

SUMMARY

1. The variety of subjects and methods heretofore used for the determination of laxative activity have been critically reviewed. The rhesus monkey was found to be the best test animal because its response to the various laxatives closely resembles that in man.

2. The earlier hypotheses on the relationship between structure and activity of laxatives of organic nature have been reviewed.

3. The laxative potencies of 36 compounds, comprising phthaleins, phthalein derivatives, and hydroxyanthraquinones, were determined on rhesus monkeys.

4. An attempt was made to find a relationship between chemical structure and laxative activity of the 36 compounds. While no definite law can be stated, the compounds showing distinct cathartic properties were found to have two phenolic hydroxyls and at least three aromatic rings in their molecule.

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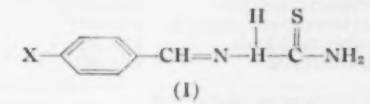
Dithiobiuret Analogs of Tibione*,†

By WILLIAM O. FOYE and JOHN J. HEFFERREN‡

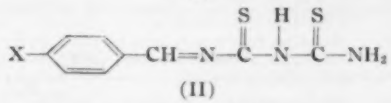
A method for preparing mono-substituted dithiobiuret derivatives of aldehydes has been developed. Compounds analogous to the aldehyde thiosemicarbazones have been prepared in fair yield and have been shown to exhibit antitubercular activity *in vitro*.

THE announcement by Domagk (1) of the promising antitubercular activity of the *para*-substituted benzal thiosemicarbazones (I), of which Tibione was the most successful example, has provoked a great deal of synthesis of related heterocyclic structures. Much less attention has been given to the effect of modification of the side-chain, however, the majority of groups such as thiosemicarbazone, semicarbazone, or carbonamide being either neutral or basic. It is the purpose of this investigation to prepare analogous structures having an acidic side-chain for comparison of antitubercular activity.

Use of the dithiobiuret group was made to provide compounds of this nature (II). Phenyl derivatives of dithiobiuret had been made as early as 1870 by Glutz (2), who reacted perthiocyanic acid with aniline, but benzal derivatives have not previously been prepared. Underwood and Dains (3) have also synthesized phenyl dithiobiurets by thiohydrolysis of pseudodithiobiurets, which are S-alkylated dithiobiurets prepared from pseudothioureas and mustard oils. Sperry (4) has condensed dithiobiuret with formaldehyde to give the disubstituted condensation product, 1,5-*bis*-(hydroxymethyl)-dithiobiuret, and cyclohexanone has likewise been condensed with dithiobiuret to give the dicyclohexylidine derivative (5), but condensations with other aldehydes or ketones have not been reported.



(I)



(II)

PROCEDURE

The major difficulty encountered in the condensation of benzaldehyde with dithiobiuret was the choice of a suitable solvent, since dithiobiuret is only slightly soluble in most organic solvents. High-boiling solvents such as nitrobenzene caused partial decomposition, and in low boiling media the reaction was unsuccessful. The use of acidic or basic catalysts either caused the liberation of sulfur or exerted no effect, although the presence of acid definitely favored condensation. When the reaction was conducted in glacial acetic acid, however, using an excess of the aldehyde, fairly good yields of the mono-condensation product could be obtained. No attempt was made to analyze the mother liquors for the amount of di-condensation products, but analytical results of some impure products indicated their presence.

Like most other aldehyde condensations, an equilibrium state was reached, and the use of aldehyde reagents was not successful in determining the extent of reaction. The use of ion exchange or adsorption media for isolation of the mono-condensation product was also ineffective because of the lack of a suitable eluting solvent. However, by using an excess of aldehyde ranging from one-half to two moles, and after one or more recrystallizations from glacial acetic acid, reasonable yields of pure mono-condensation product could be obtained. The products prepared are listed in Table I.

The results of *in vitro* tests[1] against human tubercle bacilli (strain $H_{37}Rv$) of the dithiobiurets are shown in Table II, where the amount of compound in milligrams per cent necessary for inhibition of growth of the organisms after three weeks is expressed. Unlike the benzal thiosemicarbazones, the *para*-substitution of electropositive groups in the molecule removed, rather than enhanced, antitubercular activity.

EXPERIMENTAL

***p*-Methoxybenzaldehyde.**—The general procedure of Buck (6) for veratraldehyde was followed for making this compound. A yield of 93% of methylated *p*-hydroxybenzaldehyde was obtained, melting at 0°, which agrees with the reported value (7).

***p*-Bromobenzaldehyde.**—This compound was prepared by the ultraviolet bromination of *p*-bromotoluene according to the procedure of Coleman and Honeywell (8). Yields of 30% were obtained of product melting at 56–57°.

***p*-Aminobenzaldehyde.**—Beard and Hodgson's procedure (9) was followed, involving the simultaneous oxidation-reduction of *p*-nitrotoluene. Yields of 65% were obtained of product melting at 70°, which agrees with the reported value (10).

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† Abstracted from a thesis presented to the Graduate School of the University of Wisconsin by John J. Hefferren in partial fulfillment of the requirements for the degree of Master of Science.
‡ Fellow of the American Foundation for Pharmaceutical Education.

[1] We are indebted to Drs. K. K. Chen and W. B. Sutton of the Lilly Research Laboratories for this information.

TABLE I.—DITHIOBIURET DERIVATIVES OF ALDEHYDES

Aldehyde Component	M. P.,[a] °C.	Yield, %	Color	Formula	Nitrogen Analysis, % Calcd.	Found[b]
Benzaldehyde	236–238	32	Pale yellow	$C_9H_9N_3S_2$	18.90	19.31
p-Methoxybenzaldehyde	239–240	39	Pale yellow	$C_{10}H_{11}ON_3S_2$	16.65	16.61
p-Bromobenzaldehyde	235–236	10	Pale pink	$C_9H_8BrN_3S_2$	13.90	13.93
p-Acetylaminobenzaldehyde	249–250	25	Orange	$C_{11}H_{12}ON_4S_2$	20.07	20.46
p-Nitrobenzaldehyde	230 (dec.)	31	Red	$C_9H_8O_2N_4S_2$	20.96	20.84
Propionaldehyde	240–241	17	Colorless	$C_6H_9N_3S_2$	23.97	24.09

[a] All melting points are corrected.
[b] The nitrogen analyses were carried out by the semimicro Kjeldahl procedure.

TABLE II.—IN VITRO ACTIVITIES AGAINST M. TUBERCULOSIS $H_{37}Rv$

Dithiobiuret Derivative	Inhibiting Concn., Mg. % 0.2	0.02	0.002	Control
Propional	−	+++	++++	++++
Benzal	−	++++	.....	++++
p-Methoxybenzal	++++	.....	.....	++++
p-Bromobenzal	ppt.	++	++++	++++
p-Acetylaminobenzal	ppt.	++++	.....	++++
p-Nitrobenzal	ppt.	−	++++	++++

***p*-Nitrobenzaldehyde.**—This compound was prepared by the acetylative oxidation of *p*-nitrotoluene according to Lieberman and Connor (11). Yields of 65% were obtained; m. p., 105–106°.

Aldehyde Condensations with Dithiobiuret.—Fifteen grams (0.11 mole) of dithiobiuret (recrystallized from water) was dissolved in 200–400 cc. of hot glacial acetic acid and treated with 0.14 to 0.22 mole of aldehyde. The mixture was refluxed for twelve to eighteen hours and then allowed to cool until crystals appeared. In the case of propionaldehyde, a temperature of 50° was more productive, and with *p*-aminobenzaldehyde a temperature of 75° was maintained for eight hours. The latter compound also underwent complete acetylation during the reaction. The finely crystalline product was filtered, washed with dilute alcohol, recrystallized from glacial acetic acid, and dried in a vacuum oven at 75°. The compounds obtained were sufficiently pure for elemental analysis.

The dithiobiuret condensation products were found to be insoluble in water and only slightly soluble in most organic solvents. The compounds were fairly soluble in hot glacial acetic acid, with the exception of the *p*-nitrobenzal- and *p*-acetylaminobenzal-derivatives which were only sparingly soluble. All the products were soluble in dilute sodium hydroxide solution, but insoluble in 10% sodium bicarbonate and dilute hydrochloric acid. Dithiobiuret itself is only slightly soluble in water and most organic solvents.

SUMMARY

1. A new type of aldehyde derivative has been prepared by effecting a condensation between aldehydes and dithiobiuret. Mono-aldehyde dithiobiurets were prepared with benzaldehyde, *p*-methoxybenzaldehyde, *p*-bromobenzaldehyde, *p*-aminobenzaldehyde, *p*-nitrobenzaldehyde, and propionaldehyde. High-melting products of good crystalline form were obtained.

2. A majority of the aldehyde dithiobiurets prepared were found to possess antitubercular activity *in vitro*.

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A Study of the Relationship of Viscosity to Free Menthol Content of Oregon and Washington Peppermint Oils*

By LOUIS FISCHER,† RAYMOND J. KAHL,‡ and NATHAN A. HALL§

Peppermint oils from two Pacific Northwest growing areas were studied as to the relationship between free menthol content and viscosity. Correlation was found in oils from the same area but could not be carried over to oils produced in a different area. An existing method for use in determining free menthol was modified to be useful for peppermint oils from this region, even though the oil samples are not taken in the field at the time the oil is distilled. Viscosity changes in refrigerated oil samples were noted.

ALTHOUGH it is not necessarily a measure of the value of peppermint oil as a flavor, the menthol content of medicinal peppermint oils must be controlled to meet the quality standards of the U. S. P. As an aid to the producer and the buyer, Swift and Thornton (1) have developed a method for determination of the free menthol in midwestern peppermint oil based upon the viscosity of the oil. This method has been of value in the field when used on freshly distilled oils or on oils which have undergone almost no oxidation.

In recent years the Pacific Northwest has assumed an increasingly important role in peppermint oil production, and today the amount of oil from this section of the United States exceeds that from any other area (2). Because Oregon and Washington oils exhibit properties somewhat different from those of oils produced in other regions, a study was undertaken of the relationship between free menthol content and viscosity of these oils to determine whether the Swift and Thornton viscosimetric method could be used or modified to be useful.

EXPERIMENTAL

Sample Selection.—Samples of oils from eastern Washington (designated as type K) and from the central section of the Willamette Valley in Oregon (designated as type S) were selected because of their different characteristics as shown by Tornow and Fischer (3). Thirty samples of each type were taken from commercial lots of oil delivered to the warehouse in 400-pound drums. No selection as to the exact age of the oil was made, although all oils were from the 1951 mint crop and were from one to two months old when the samples were taken. It was realized that changes had taken place which might have affected the viscosity of the oil. However, the study was intended to show whether viscosimetric measurements could be applied to these oils which had received no special handling. Samples were kept in tightly stoppered glass containers at 10° until used in the investigation.

Chemical Determination of Free Menthol.—Esters and total menthol were determined by the U. S. P. XIV method (4), modified slightly by the addition to the sample of 20 ml. of distilled water just before titration of the excess alcoholic potassium hydroxide and an additional 10 ml. near the end point. This procedure gave a much more distinct color change with the phenolphthalein indicator and better reproducibility. Free menthol was calculated by subtraction of the menthol present as esters from the total menthol.

Viscosimetric Measurements.—The viscosity was measured by the method of Swift and Thornton (1). Samples of the oil (5 ml.) were allowed to run through an Ostwald pipette and the time required in seconds was recorded. All measurements were made at 30° with the pipette immersed in a thermostatically controlled water bath. In each case the reading recorded was an average of 3 runs, and the same 2 calibrated pipettes were used for all measurements. To determine the stability of the oils on storage, viscosity measurements were made immediately when the samples were obtained and again at the end of four months' storage at 10°. No conversion of time measurements to absolute viscosity values was made.

Preparation of Standard Curves.—Standard curves were prepared by 2 methods: first, by the method of Swift and Thornton (1) in which the menthol-poor fraction obtained on steam distillation of the oil was fortified with pure *l*-menthol; and second, by fortifying the oil of the series containing the lowest per cent of free menthol with *l*-menthol. Fortification was carried out in about 5% increments and the viscosity of the resulting solution was measured. The viscosity in seconds was plotted against the free menthol content.

Viscosimetric Determination of Free Menthol.—The free menthol content of each oil was obtained by measuring the viscosity, and the per cent free menthol was read directly from the above standard curves, except that in case of those curves prepared by the Swift and Thornton method, their "turbidity

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§ The authors wish to express their appreciation to Mr. Paul A. Tornow of I. P. Callison and Sons, Chehalis, Wash., for supplying the oil samples which were used in this investigation.

correction factor" was applied. This factor reflected the various degrees of turbidity obtained by mixing one volume of the oil with 4 volumes of a solvent containing equal volumes of methanol and 70% ethanol.

RESULTS AND DISCUSSION

Effect of Storage on Viscosity.—Reitsma and Cramer (5) in a recent publication show that the viscosity increase of peppermint oil parallels the oxidation of the oil. They demonstrate further that this effect can be minimized if the oil is stored at low temperatures and in tightly closed, full containers. Our results are summarized in Table I; one sample of type S oil is omitted from the data since it showed a viscosity increase of sixty-seven seconds.

TABLE I.—VISCOSITY CHANGES OF PEPPERMINT OIL WHEN STORED FOR FOUR MONTHS AT 10°

Type of Oil	No. of Samples	Max. Increase, Sec.	Min. Increase, Sec.	Av. Increase, Sec.
S (Western Oregon)	29	22	0	7
K (Eastern Washington)	30	11	0	4

These values differ from the results of Reitsma and Cramer (5) who report that eastern Washington oil is much more prone to oxidation than either midwestern United States or western Oregon and Washington oil. This difference may be accounted for by the assmption that the oils used in this investigation had undergone some oxidation before the samples were taken. If this assumption is correct, the eastern Washington oils would tend to reach an oxidation equilibrium more rapidly than western Oregon oils and would be more stable to subsequent oxidation on storage. This is born out by the experimental data.

Viscosimetric Determination of Free Menthol.—Results obtained from standard curves prepared by the method of Swift and Thornton and by fortification of a natural peppermint oil with pure *l*-menthol are summarized in Tables II and III. Turbidity corrections are applied to the results obtained by the method of Swift and Thornton but not to the results obtained by the modification of their method.

Western Oregon (type S) peppermint oil shows correlation between free menthol content and viscosity when the standard curve is constructed by either of the methods. The relationship is somewhat more quantitative when the standard curve is prepared from the viscosity values obtained from a natural oil fortified with menthol rather than from a menthol-poor fraction of an oil fortified with menthol.

TABLE II.—VISCOSIMETRIC DETERMINATION OF FREE MENTHOL IN TYPE S (WESTERN OREGON) PEPPERMINT OILS

Sample No.	Free Menthol By Chemical Method, %	Free Menthol By S and T Method, %	Difference Between Chemical and S and T Methods, %	Free Menthol By Modified Method, %	Difference Between Chemical and Modified Methods, %
1	46.6	45.2	−1.4	45.1	−1.5
2	42.9	43.1	+0.2	42.4	−0.5
3	45.5	46.2	+0.7	46.4	+0.9
4	47.4	45.6	−1.8	45.6	−1.8
5	42.2	40.3	−1.9	41.8	−0.4
6	45.5	42.0	−3.5	44.2	−1.3
7	46.2	45.0	−1.2	47.4	+1.2
8	45.5	43.0	−2.5	44.7	−0.8
9	48.3	45.7	−2.6	48.4	+0.1
10	40.4	39.7	−0.7	40.8	+0.4
11	47.5	45.4	−2.1	47.9	+0.4
12	42.9	41.5	−1.4	43.0	+0.1
13	46.8	44.2	−2.6	46.3	−0.5
14	42.0	40.6	−1.4	41.7	−0.3
15	47.6	45.0	−2.6	47.6	0.0
16	44.4	42.3	−2.1	44.0	−0.4
17	45.5	44.3	−1.2	46.5	+1.0
18	49.2	47.9	−1.3	51.2	+2.0
19	45.2	43.7	−1.5	45.9	+0.7
20	45.9	45.0	−0.9	47.4	+1.5
21	44.1	41.9	−2.2	43.7	−0.4
22	44.2	42.8	−1.4	44.5	+0.3
23	46.4	45.4	−1.0	48.0	+1.6
24	48.2	46.2	−2.0	48.9	+0.7
25	42.8	41.5	−1.3	42.1	−0.7
26	43.1	42.3	−0.8	43.0	−0.1
27	42.5	41.1	−1.4	41.6	−0.9
28	44.3	42.8	−1.5	44.5	+0.2
29	46.7	51.3	+4.6	51.7	+5.0
30	42.5	45.0	+2.5	47.5	+5.5
Maximum Difference		4.6			5.5
Minimum Difference		0.2			0.0
Average Difference[a]		1.5			0.7

[a] Samples 29 and 30 omitted in calculation of average.

TABLE III.—VISCOSIMETRIC DETERMINATION OF FREE MENTHOL IN TYPE K (EASTERN WASHINGTON) PEPPERMINT OIL

Sample No.	Free Menthol By Chemical Method, %	Free Menthol By S and T Method, %	Difference Between Chemical and S and T Method, %	Free Menthol By Modified Method, %	Difference Between Chemical and Modified Methods, %
1	42.5	35.3	−7.2	41.4	−1.1
2	41.3	33.5	−7.8	40.5	−0.8
3	29.0	29.5	+0.5	29.0	0.0
4	42.1	34.7	−7.4	40.6	−1.5
5	48.4	39.5	−8.9	47.8	−0.6
6	47.9	38.2	−9.7	47.4	−0.5
7	38.7	32.0	−6.7	37.1	−1.6
8	46.7	38.2	−8.5	47.3	+0.6
9	41.5	33.5	−8.0	40.5	−1.0
10	39.6	27.8	−11.8	38.4	−1.2
11	33.6	29.7	−3.9	35.1	+1.5
12	46.1	38.7	−7.4	47.6	+1.5
13	32.3	29.7	−2.6	30.3	−2.0
14	40.1	33.3	−6.8	38.5	−1.6
15	39.6	33.0	−6.6	38.2	−1.4
16	38.8	32.2	−6.6	38.3	−0.5
17	40.4	32.5	−7.9	38.5	−1.9
18	45.4	36.7	−8.7	44.9	−0.5
19	38.0	31.5	−6.5	37.5	−0.5
20	39.6	31.8	−7.8	38.0	−1.6
21	35.8	32.7	−3.1	36.3	+0.5
22	36.8	31.5	−5.3	37.4	+0.6
23	40.1	34.3	−5.8	38.3	−1.8
24	39.2	33.8	−5.4	37.8	−1.4
25	40.8	32.2	−8.6	38.9	−1.9
26	38.6	32.5	−6.1	38.5	−0.1
27	37.6	31.2	−6.4	36.8	−0.8
28	37.1	31.5	−5.6	37.3	+0.2
29	43.0	33.7	−9.3	40.0	−3.0
30	42.3	38.5	−3.8	45.9	+3.6
Maximum Difference		11.8			3.6
Minimum Difference		0.5			0.0
Average Difference		6.7			1.2

Eastern Washington (type K) peppermint oil shows poor correlation between free menthol content and viscosity when the standard curve is prepared by fortification of a menthol-poor fraction of an oil. However, if a natural K oil is fortified with menthol and a standard curve is prepared from the oil thus obtained, correlation is evident. The standard curves for oils from each area are not interchangeable. Erroneous results are obtained when viscosity readings of type K oil are applied to a standard curve made from a type S oil.

Two of the 30 oils from each area fail to give satisfactory results with either method. The explanation of this fact is unknown by the authors, for in appearance and organoleptic properties these oils are similar to the others in the series. It is felt that the data on these 4 oils represent an important consideration in the practical application of the viscosimetric determination of free menthol because they place emphasis on a limitation of the method.

These results show that, in a given growing area with oils of approximately the same age which have been stored under similar conditions, the viscosimetric method for determination of free menthol may be used for control purposes with good possibilities of success. These conditions would be met normally when oil from a single year's crop of mint comes from the field and is placed in the warehouse of the buyer before blending or rectifying.

SUMMARY

A viscosimetric method for the determination of free menthol in peppermint oil, developed for oils grown in the midwestern United States, has been applied to Pacific Northwest peppermint oils from two growing areas. Usable correlation between free menthol content and viscosity was obtained when the method of preparing the standard curve was modified by fortifying a natural oil of low menthol content (rather than a rectified oil) with pure *l*-menthol. The method was found to have limitations.

The change in viscosity occurring in oils stored in a refrigerator in tightly stoppered containers is discussed.

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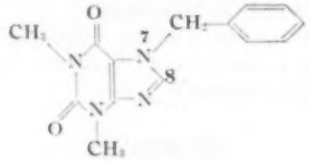
Theophylline Derivatives. I. Analogs of 2-Benzyl-2-Imidazoline (Priscoline)*,†

By GEORGE P. HAGER, JOHN C. KRANTZ, Jr., and JOHN B. HARMON

8-Benzyltheophylline is analogous from a structural standpoint to the synthetic adrenergic-blocking agent, Priscoline, and was prepared along with various related compounds with the hope that the resulting hybridization of structural characteristics would result in an agent useful clinically in treatment of hypertension. The following compounds are reported: 8-benzyltheophylline; 8-(2-methylene-2-imidazoline)-theophylline; 7-benzyltheophylline; and 7-(2-methylene-2-imidazoline)-theophylline. 8-Benzyltheophylline in preliminary pharmacologic tests showed promise of being a potent hypotensive agent, and current studies have as their objective an elaboration of the structure of 8-benzyltheophylline in order to provide a basis for correlation of structure and activity.

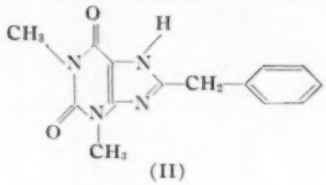
THE USE of theophylline clinically for the control of the symptoms of hypertension suffers from disadvantages of irregular absorption and undesirable side effects in the gastrointestinal tract (1). Such disadvantages have been overcome to a certain extent by pharmaceutical measures including use of the product of interaction of theophylline with ethylenediamine (aminophylline, U. S. P. XIV), sodium glycinate [Theoglycinate (2)] or sodium acetate (theophylline and sodium acetate, N. F. IX). Compounds which represent a fundamental modification of the structure of theophylline, especially with regard to the substituent at positions 7 or 8 (see Formula I), have been reported from time to time (3–5), but the pharmacologic properties of these compounds (as far as their effect on blood pressure is concerned) have not been included in these reports.

Theophylline can be substituted readily in the 7-position, because of the acidic character of the hydrogen, by reactions involving the nucleophilic anion of the potassium salt of theophylline. By the reaction of benzyl chloride with the potassium salt of theophylline, 7-benzyltheophylline (I) can be prepared (6); the same is true of a



(I)

number of related compounds (3–5). The isomeric 8-benzyltheophylline (II) was of considerably greater interest in this study of hypotensive theophylline derivatives because of the structural features which it possesses in common with Priscoline (III). Substitution of the imidazole



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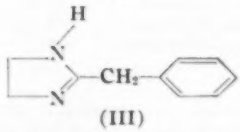
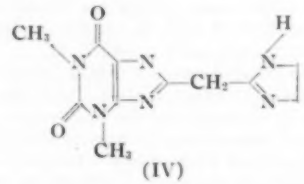


(III)

grouping of the theophylline molecule in the 8-position (8-benzyltheophylline) bore an interesting analogy to the substitution of imidazoline in the 2-position (2-benzyl-2-imidazoline or Priscoline).



(IV)

It was hoped that this hybridization of the structural characteristics of theophylline and Priscoline might be achieved without sacrificing the vasodilating properties of the former or the adrenergic-blocking activity of the latter, since both types of pharmacodynamic activity are indicated in the clinical control of hypertension.

Since the 2-methylene-2-imidazoline grouping may be the important determinant of the adrenergic-blocking activity of Priscoline, 8-(2-methylene-2-imidazoline)-theophylline (IV) and the isomeric 7-(2-methylene-2-imidazoline)-theophylline were also prepared.

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† Abstracted from a thesis presented to the Graduate School of the University of Maryland by John B. Harmon in partial fulfillment of the requirements for the degree of Master of Science.

Substitution of the 7-position of theophylline was accomplished by the reaction of the potassium salt of theophylline with the appropriate halide. Theophylline-7-acetic acid was prepared in this way (7) and, after esterification, was converted to 7-(2-methylene-2-imidazoline)-theophylline (Eq. 1) by treatment with ethylenediamine according to a known procedure for the preparation of imidazolines (8).

The use of benzyl chloride in place of chloroacetic acid resulted in the formation of 7-benzyltheophylline.

1,3-Dimethyl-5,6-diaminouracil, prepared essentially as directed by Traube (9), using sodium hydrosulfite instead of ammonium sulfide for reduction of 1,3-dimethyl-6-imino-5-isonitrosouracil (10), was a useful intermediate for the preparation of 8-substituted theophyllines. Fusion of this diamine with a carboxylic acid or derivative produced first an amide and finally the 8-substituted theophylline as indicated by Eq. 2.

When cyanoacetic acid was used in this reaction theophylline-8-acetic acid was produced. This intermediate was converted to 8-(2-methylene-2-imidazoline)-theophylline by the reaction of the corresponding ester with ethylenediamine as described above.

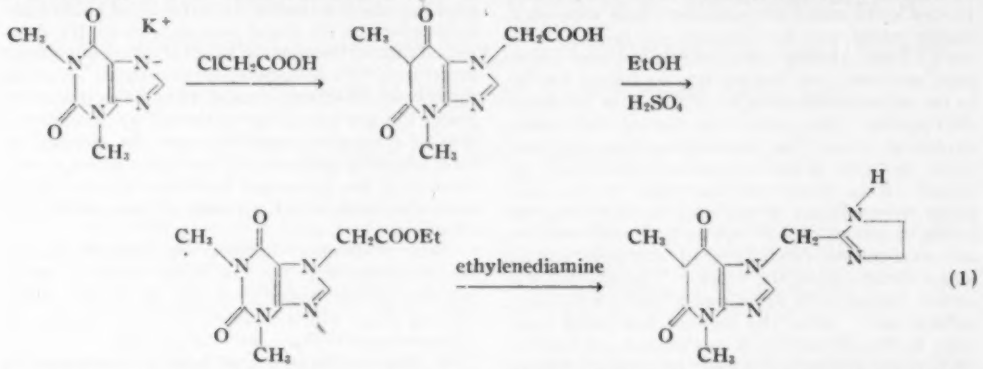
Since 8-benzyltheophylline in preliminary pharmacologic tests showed promise of being a potent hypotensive agent, current studies have as their objective an elaboration of its structure, in order to provide a basis for correlation of structure with activity.

EXPERIMENTAL

All melting points reported were measured with a Fisher-Johns apparatus and are uncorrected.

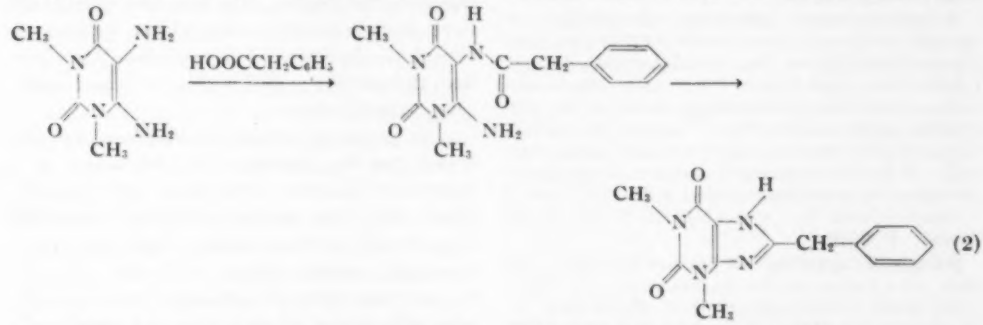
7-Benzyltheophylline.—The potassium salt of theophylline was prepared by dissolving 10 Gm. (0.05 mole) of theophylline in 200 cc. of 95% ethanol with gentle heating, adding 100 cc. of 0.5 *N* alcoholic potassium hydroxide, and filtering the precipitated salt from the chilled solution. The salt was washed with alcohol and ether and dried.

In an 8-in. Pyrex test tube were placed 2 Gm. (0.009 mole) of the potassium salt of theophylline, 1.1 Gm. (0.009 mole) of benzyl chloride, and 5 cc. of water. The tube containing the heterogeneous mixture was heated at 100° for six hours after sealing. The solid product was recrystallized twice from water, and 1.5 Gm. (60% of theoretical) of 7-benzyltheophylline was obtained as long silky needles, m. p. 154–155°, reported m. p. 158° (6).



(1)

Theophylline-7-acetic Acid.—This compound was prepared by the method described by Baisse (7). Yields as high as 88% after recrystallization from water were obtained; m. p. 263–266°, reported m. p. 260°.



(2)

Ethyl Theophylline-7-acetate.—A mixture of 25 Gm. (0.1 mole) of theophylline-7-acetic acid, 100 cc. of absolute ethanol, and 50 cc. of sulfuric acid (added dropwise with stirring) was warmed until homogeneous and was allowed to stand overnight

in the refrigerator. By recrystallization from water of the solid which precipitated from the solution, 18.1 Gm. (74% of theoretical) of ethyl theophylline-7-acetate was obtained; m. p. 140–141°.

Anal.—Calcd. for $C_{11}H_{14}N_4O_4 \cdot H_2O$: N, 19.71. Found: N, 20.36.

7-(2-Methylene-2-imidazoline)-theophylline.—A mixture of 10 Gm. (0.035 mole) of ethyl theophylline-7-acetate and 9 Gm. (0.15 mole) of freshly distilled ethylenediamine was heated in a sealed tube for thirty-two hours at 100°. Excess ethylenediamine was removed by distillation under reduced pressure. The residue was recrystallized from isobutyl alcohol, and 7.1 Gm. (72% of theoretical) of 7-(2-methylene-2-imidazoline)-theophylline was obtained; m. p. 198–200° (dec.).

Anal.—Calcd. for $C_{11}H_{14}N_6O_2 \cdot H_2O$: C, 47.13; H, 5.75; N, 29.99. Found: C, 47.43; H, 5.94; N, 29.34.

Ethyl Theophylline-8-acetate.—Ten grams of 1,3-dimethyl-5,6-diaminouracil (0.06 mole) and 10 Gm. (0.12 mole) of cyanoacetic acid were thoroughly mixed, and the mixture was fused at 115–120°. Upon heating for a short time, the molten mass solidified, and heating was continued for five to ten minutes to remove all of the water formed in the reaction. The product was treated with boiling alcohol to remove the excess cyanoacetic acid, and about 10.8 Gm. of the intermediate amide was obtained. Ring closure and hydrolysis of the cyano group were effected in one step by dissolving the amide in 100 cc. of 2 *N* sodium hydroxide solution and evaporating carefully until ammonia was no longer detectable in the vapors. The solution was cooled, diluted with water, and acidified with dilute sulfuric acid. After the mixture had stood overnight in the refrigerator, it was filtered and 5.4 Gm. (36% of the theoretical amount obtainable from the 1,3-dimethyl-5,6-diaminouracil employed) of theophylline-8-acetic acid was obtained.

A solution of 5 Gm. (0.02 mole) of theophylline-8-acetic acid in 50 cc. of absolute ethanol was saturated with dry hydrogen chloride. Ethyl theophylline-8-acetate crystallized from the chilled mixture and was washed with alcohol and ether and dried; yield, 5.5 Gm. (97% of theoretical); m. p. 216–218°, reported m. p. 215° (5).

8-(2-Methylene-2-imidazoline)-theophylline.—A mixture of 6.3 Gm. (0.02 mole) of ethyl theophylline-8-acetate and 15 Gm. of ethylenediamine was refluxed for eight hours. The excess ethylenediamine was removed as completely as possible by distillation under reduced pressure and the residue was recrystallized twice from 95% ethanol, using charcoal. The 8-(2-methylene-2-imidazoline)-theophylline obtained in this way melted at 224–226° (dec.).

Anal.—Calcd. for $C_{11}H_{14}N_6O_2 \cdot H_2O$: N, 29.55. Found: N, 28.88.

8-Benzyltheophylline.—A mixture of 5 Gm. (0.03 mole) of 1,3-dimethyl-5,6-diaminouracil and 5 Gm. (0.04 mole) of phenylacetic acid was heated by means of a hot plate. The mixture melted at 80° and resolidified at 120°. The solid obtained in this way was recrystallized from ethanol and found to melt at 235–240°. This material, which was probably the intermediate amide, was dissolved in hot sodium hydroxide solution, and 8-benzyltheophylline precipitated from the solution upon acidification. The product was purified by dissolving in a calculated amount of 5% NaOH solution (using heat), decolorizing the solution with charcoal, filtering, and saturating with carbon dioxide. It can also be purified by recrystallization from pyridine. By this means, 5.8 Gm. (70% of theoretical) of 8-benzyltheophylline was obtained; m. p. 286–287°.

Anal.—Calcd. for $C_{14}H_{14}N_4O_2$: N, 20.21. Found: N, 20.09.

PHARMACOLOGY[1]

Of the four theophylline derivatives prepared in this investigation, 8-benzyltheophylline was the most active in producing a fall in blood pressure. In preliminary tests on several dogs, the compound lowered the blood pressure to 40–50% of normal in doses of about 13 mg./Kg. A dose of 6 mg. of 8-benzyltheophylline per Kg. caused a 25% fall in blood pressure, while the same dose of Theoglycinate produced only a 5% fall. Atropine did not modify the vasodepressor effect of 8-benzyltheophylline, and it is concluded that it is not cholinergic in its effect on the blood pressure. Since the compound further lowered the blood pressure of animals pretreated with histamine, apparently its action is directly on the arterial musculature. Electrocardiograms did not appear to be altered by administration of 8-benzyltheophylline, and the increase in tone following perfusion of the frog's heart with a solution of the compound indicates that the fall in blood pressure is not a result of any cardiotoxic effects.

These preliminary studies also showed that 8-benzyltheophylline has an acute toxicity when injected intraperitoneally in the rat (LD_{50}: 300–350 mg./Kg.) which is about the same as that of Theoglycinate (LD_{50}: 300–400 mg./Kg.).

In these preliminary experiments, no evidence of adrenergic-blocking activity was obtained, and the objective of combining the pharmacodynamic properties of Priscoline with theophylline was not achieved.

SUMMARY

1. With the primary objective of finding a hypotensive agent free from the undesirable side effects of theophylline, the following compounds have been prepared: 7-benzyltheophylline; 8-benzyltheophylline; 7-(2-methylene-2-imidazoline)-theophylline; and 8-(2-methylene-2-imidazoline)-theophylline.

2. In preparing these compounds, it was also hoped that the adrenergic-blocking action of 2-benzyl-2-imidazoline (Priscoline) may be combined with the pharmacodynamic properties characteristic of theophylline. Preliminary pharmacologic studies indicate that this objective has not been achieved, although the compounds, especially 8-benzyltheophylline, are structurally analogous to Priscoline to some extent.

3. The results of the preliminary pharmacologic studies of 8-benzyltheophylline were sufficiently

[1] The pharmacologic studies are being carried out by Dr. John C. Krantz, Jr., and will be reported in detail in a later publication.

encouraging to warrant further investigation of its pharmacologic properties and those of other 8-substituted theophyllines. Such an investigation is now in progress.

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Studies on *Veratrum*. II. *Veratrum Eschscholtzii* A. Gray; Observations on Seed Germination and Early Growth of Seedlings of *Veratrum* Species*

By HEBER W. YOUNGKEN†, ‡

A report is presented of studies made on the structure, habit, and distribution of *Veratrum Eschscholtzii* A. Gray, and a preliminary report of tests made on the germination of *Veratrum* seeds and the culture of the seedlings is given. A higher yield of *Veratrum* seedlings resulted when seeds were given refrigeration at 32–35° F. for a period of one to two months, then dusted with Arasan or Semesan, and planted in pots or flats containing a mixture of washed fine sand, loam, and horticultural peat moss.

IN A PREVIOUS paper in THIS JOURNAL (1), the results of studies of the roots of a number of different species of *Veratrum* were discussed.

The purpose of the present report is to present the results of further studies on the fresh and dried plants of *Veratrum Eschscholtzii*, including their habitat, distribution and structure, and the anatomy of the rhizome and roots; and to give a preliminary report on seed germination tests and seedlings of *Veratrum viride* and *V. album*.

The dried rhizomes and roots of *Veratrum Eschscholtzii* are now being investigated chemically and have already been reported to contain a number of the same alkaloids found in *Veratrum viride*, namely, jervine, rubijervine, pseudojervine, and veratramine (2).

Many resemblances occur between these two species, but there are still a few recorded differences, a fact which has caused uncertainty among botanists and pharmacognosists as to whether *Veratrum Eschscholtzii* is a distinct species or a western variety or form of *V. viride* Aiton. It is hoped that the observations recorded in this report will help to solve the problem.

EXPERIMENTAL

Materials and Methods

The materials used in this investigation consisted of living young plants of *Veratrum Eschscholtzii* collected for the author by Dr. Carl A. Taylor at Juneau, Alaska, shipped by air express, and transplanted to the medicinal plant garden of the Massachusetts College of Pharmacy and Waltham Field Station, where their growth habits were studied; from these plants, materials were gathered for detailed laboratory study. Also used were two entire dried rhizomes and roots and about 3 lb. of dried rhizomes and roots in segments supplied by the Riker Laboratories, and numerous herbarium sheets of *V. Eschscholtzii* gathered by different botanists in Alaska, British Colombia, Washington, Montana, and Oregon, and deposited in the Gray Herbarium.

The living plant materials and the herbarium sheets were compared with published descriptions of the species by Gray (3) and Rydberg (4) and authenticated by the author.

The seeds used in germination studies were obtained from plants of *Veratrum viride* growing in New Hampshire and from *Veratrum album* as supplied by seedsmen in Europe.

The flowers and other parts of the inflorescences of *Veratrum Eschscholtzii* and of *V. viride* were ex-

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amined under a binocular dissecting microscope, and the stamens, perianth lobes, pedicels, and bracts were measured. The surfaces of the leaves of both species were compared for hairiness. Temporary mounts in water, chloral hydrate solution, phloroglucin-HCl, and lactophenol were made separately of sections of the rhizomes and roots of both species. Schulze's maceration method was employed in preparing entire tissue elements for microscopic examination. The powdered drug was examined in water mounts for the study of the starch grains and crystals and in phloroglucin-HCl for the examination of endodermis, vascular, and other hard-walled elements.

Transverse and radial longitudinal sections of the rhizomes and roots of different plants of *V. Eschscholtzii* and *V. viride* were cut, cleared of starch, stained with safranin and fast green, dehydrated, cleared with benzol, and mounted in Canada balsam and labeled as permanent mounts. Large numbers of the temporary and permanent mounts of parts of both species were studied and compared, and observations were recorded.

Taxonomy

Uncertainty has long existed among plant taxonomists as to whether *Veratrum Eschscholtzii* A. Gray represents a distinct species or a variety or race of *Veratrum viride* or *V. album*. The Index Kewensis (5) cites it as equivalent to *Veratrum album*. Harshberger (6) named it *Veratrum viride* var. *penduliflorum*. Anderson (7), while describing it under *V. Eschscholtzii* A. Gray, called it American White Hellebore and stated that it is *V. viride* in part. Abrams (8) calls it *Veratrum viride* Ait.

Hulten (9) only provisionally accepted it as a distinct species, stating that it differs from the eastern American *V. viride* in the long drooping branches of the inflorescences, the more hairy leaves, and the somewhat longer stamens.

Gray (3) originally described the species as follows: "paniculæ ramis lateratibus plerumque nutantibus; foliolus perianthii campanulati oblongis, basi attenuatis, filamentis gracillimus plusduplo longioribus." He gave its habitat as northwestern North America. The more important taxonomic synonymy follows:

Veratrum Lobelianum β-Eschscholtzianum in *Roem. & Schult. Syst. Veg.*, **7**, 1555(1829).

Veratrum Eschscholtzii A. Gray in *Ann. Lyc. Nat. Hist. N. Y.*, **4**, 119(1837).

Veratrum parviflorum Bongard, Veg. Sitcha in *Mem. Acad. St. Petersb.* (*Ser.* 6), **2**, 166(1833).

Veratrum viride Hooker in *Flora Bor. Amer.*, **2**, 178 (1840).

Veratrum album var. *Lobelianum* Kurz in Engler, *Bot. Jahrb.*, **19**, 410(1895).

Veratrum Eschscholtzianum Rydberg in *Muhlenbergia*, **1**, 20(1905).

Veratrum viride var. *penduliflorum* Harshberger in *Acta Forest. Fenn.*, **34**, 5(1939).

Habitat and Distribution.—*Veratrum Eschscholtzii* A. Gray is a distinctly western North American perennial herb distributed from the Bering Sea district through Alaska and southward through British Colombia, the mountain sections of Washington, along river banks and on Mt. Hood in Oregon, and eastward into Montana and Idaho. According to Taylor (10) who observed it growing in the area around Juneau to Ketchikan, and in Washington and Oregon, it occurs liberally in distant spots in very wet, well-drained soil of schist origin, very low in nitrogen and in phosphorus and only moderately supplied with potassium. The *p*H ranges from 4.5 to 5.9 in the Alaskan sites and approaches 6.6 on Mt. Baker in northern Washington. In northwestern Washington, it grows between the elevation of 3,500 to 4,400 ft. In southern Washington on Mt. Adams, Taylor states, it is limited to elevations of 5,900 to 6,000 ft. and is at its best on a band of 5,500 ft. where it grows on slopes having a fairly deep, well-aerated, and well-drained soil consisting of fluffy, fine volcanic ash. On Mt. Hood in northern Oregon, the plants were found in sunny and partly shaded places at the edges of timber or in small clearings where the ground was wet and at elevations of 4,300 to 4,800 ft.

Description.—The entire plants examined by the author exhibited a subterranean portion consisting of a vertical, fleshy, deeply set rhizome bearing very long, thick, sparsely branched roots, the latter attaining a thickness greater than numerous roots of *Veratrum viride* Ait. which were examined by the writer and which were gathered in many sections of eastern United States from the southern Blue Ridge to Quebec and Ontario. The overground plants exhibited one or two stems attaining a height 1–2 m. and bearing alternate, ovate-oblong to ovate-lanceolate clasping, cauline leaves, glabrous above and usually arachnoid pubescent beneath, the lower with blades up to 31 cm. in length and up to 16 cm. in width; the uppermost, lanceolate, sessile, and sheathing at the base. The flowers are greenish and arranged in a panicle whose side and lower branches are usually drooping but sometimes ascending. The upper flowers are perfect, the lower staminate. The perianth is liliaceous and consists of 6 green, oblanceolate-to-spatulate lobes up to 8 mm. in length and up to 3 mm. in width and pubescent beneath. The stamens are up to 8 to 9 mm. in length and equal or exceed the perianth lobes; they have slender filaments and cordate anthers. The bractlets are lanceolate, up to 10 mm. long and 3 mm. wide, and hairy beneath. Peduncles, pedicels, and bractlets are puberulent. The fruit is a many-seeded capsule usually from 10 to 12 mm. in length. The seeds are flat with broad wings. The growing plants and the root system are shown in Figs. 1–3.

Veratrum Eschscholtzii and *V. viride* are without doubt very closely related plants, showing fewer differences than resemblances when large numbers of each are examined comparatively. One of the most stable differences appears to be the number of new roots produced each year. Under good soil conditions, *Veratrum Eschscholtzii* usually produces 10 to 15 while *V. viride* forms 20 to 35.

It has been asserted that the leaves of *V. Eschscholtzii* are entirely hairy on their lower surfaces while those of *V. viride* are only scantily hairy on the veins below. This is not always true. The writer has examined some *V. Eschscholtzii* leaves whose lower surfaces were similar to those of *V. viride* in scanty hairiness. The greater hairiness of the leaves of *V. Eschscholtzii* appears to pertain chiefly to the plants growing in Alaska and northern Washington and could be attributed to differences in temperature, climate, and possibly other ecological conditions.

Fig. 1.—*Veratrum Eschscholtzii* A. Gray. Flowering and vegetative plants.

Veratrum Eschscholtzii is asserted to possess panicles with long drooping branches (11) whereas the panicle branches of *V. viride* are stated to be ascending. However, this character, which is usually tenable as distinctive, is not inviolate, for we have examined panicles of both species possessing both drooping and ascending side branches, the latter arching near the tip.

Veratum Eschscholtzii Rhizomes and Roots

Unground Drug.—The commercial material occurs in transverse and oblique segments of the rhizome with attached broken roots and as entire rhizomes and roots. The entire dried rhizomes are vertical, up to 8 cm. in length and up to 3.5 cm. in diameter; irregularly cylindrical, ovoid to conical in shape; externally grayish brown to dark brown or black; the upper, anterior end with numerous circular remains of leaf sheaths, a stem base or scar and/or one or two conical buds, the lateral surface surrounding the rhizome annulate and with numerous long tapering rootlets and scattered root scars of circular outline, the latter alone appearing in the posterior region; internally white or pale yellow, and showing a wavy yellow endodermis separating the narrower cortex from the broad central cylinder. The dried rootlets are up to 15 cm. in length and up to 5 mm. in diameter at the base, elongated, conical, and tapering; externally yellowish to yellowish-brown and showing numerous, closely-set transverse wrinkles and some irregularly longitudinal wrinkles and furrows. The fracture is short, the fractured surface white and starchy. The odor is indistinct, the taste bitter and acrid.

Anatomy of the Rhizome.—In its general arrangement of tissues, the rhizome of *V. Eschscholtzii* resembles that of *V. viride*.

Transverse sections cut at different levels are circular with a wavy periphery. A transverse section 20 mm. in diameter possessed a cortex of 4 mm. or

Fig. 2.—*Veratrum Eschscholtzii* A. Gray. Panicles of ripened capsular fruits.

one-fifth the total diameter. It exhibited a thin, disintegrating epidermis in the process of exfoliation; a dark brown metaderm of usually up to 5, occasionally up to 8, layers of cells with suberized walls; a cortex of starch- and crystal-bearing parenchyma traversed by the horizontal root trace bundles and oblique to cross sections of leaf trace bundles; a wavy, yellow endodermis of one to two layers of endodermal cells of quadratic, irregularly quadratic to oval-shaped cells, mostly with U-shaped lumina and thickened on their radial and inner walls, some with nearly uniform thickening on the radial, inner, and outer walls. The endodermis is broken in places for the passage of the root and leaf traces. It is only partially lignified in its walls. Beneath the endodermis is the broad central cylinder consisting of a matrix of starch- and crystal-bearing parenchyma through which course numerous stem bundles. The outer ring of bundles beneath the endodermis are of closed collateral type and closely set. The remaining bundles are larger, of the amphivasal type, and scattered through the remainder of the central cylinder. Only the root trace bundles possess fibers. Raphide-sacs with bundles of acicular crystals of calcium oxalate are scattered through both cortex and central cylinder.

Longitudinal-radial sections exhibited the raphide sacs arranged in axile fashion and usually end to end. They were up to 228 μ in length and contained the raphides imbedded in mucilage. The raphides in the Alaskan material were up to 135 μ in length. In lengthwise view the tracheids and vessels were of scalariform, scalariform-reticulate, pitted, and spiral types. The scalariform vessels were up to 38 μ in diameter and often exhibited numerous bulges and lobes. The endodermal cells in these sections ex-

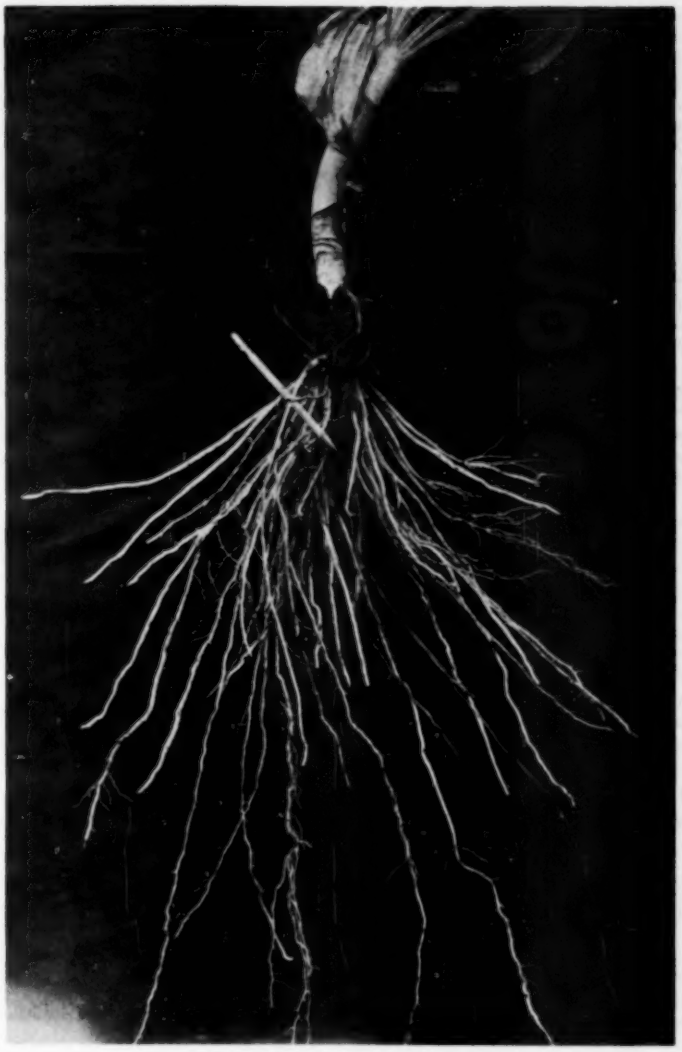

Fig. 3.—Root system of *Veratrum Eschscholtzii* showing the long, sparsely branched roots characteristic of this species. Lead pencil indicates relative size, and its point rests at the base of the rhizome. Dug at elevation of 5,350 ft., July 22, 1951, on Mt. Adams, southern Washington, by C. A. Taylor. The photograph is reproduced through the courtesy of Dr. Taylor.

hibit a variety of shapes (rhomboidal, irregularly rectangular, irregularly oval, elliptical, or rounded). They are up to 42 μ in length and up to 26 μ in width, and exhibit mostly broad U-shaped lumina. Their walls are pitted, as can be best seen in mounts of strong chloral hydrate solution. Their lumina varied greatly but were usually greater than the combined thickness of the walls. The parenchyma cells in this view ranged from 22 μ x 26 μ to 26 μ x 53 μ and contained simple and 2- to 5-compound starch grains. The individual starch grains attained a larger diameter when compared with those of *Veratrum viride* rhizomes.

Anatomy of the Root.—In addition to the characteristics previously reported by the author (12), the following were noted: in sections cut at short distances from the tip of the younger roots found just beneath the crown of the rhizome, aerenchyma was absent, but in a series of sections cut through the remaining portion of younger roots toward their bases, it gradually developed, occupying about one-third the width of the cortex in the region of maturity toward the base. In roots of previous years' growth arising at lower levels from the rhizome, aerenchyma occurred throughout most of the length of the root, its air spaces occasionly extending from hypodermis to endodermis in the upper half of these. In longitudinal radial sections, the endodermal cells are longitudinally elongated and closely beaded on their longitudinal walls, the latter varying from non-

lignified to lignified. All of the endodermal cells in cross sections showed U-shaped lumina and none exhibited V-shaped lumina as observed by the writer in some sections of *V. viride* roots. From 16 to 17 alternating xylem and phloem strands were found in radial the arrangement of basal regions of some roots.

Powdered Drug.—The powdered material of rhizomes and roots is similar in color, odor, and taste to that of *Veratrum viride* and, like it, strongly sternutatory. When suitably mounted it exhibits the following microscopic structures: numerous fragments of starch- and crystal-bearing parenchyma; numerous simple and 2- to 3-compound, fewer 4- to 5-compound starch grains, the individual grains spheroidal, oval, plano-convex, or angular-convex, usually up to 16.5 μ, occasionally up to 23 μ in diameter, with a circular, linear, crescentic, or 2- or more cleft hilum; numerous raphides of calcium oxalate up to 140 μ in length; numerous fragments of vascular tissues and wood fibers, the scalariform tracheids and vessels more numerous than the pitted and spiral types and frequently showing bulges, their ends occasionally forked; fragments of reddish-brown to dark brown metaderm; fragments of pith parenchyma of roots with lignified walls; fragments of collenchyma, epidermal, and sieve tissues; endodermal cells with U-shaped lumina and with radial and inner walls thicker than outer walls.

The starch grains and endodermal cells attained a larger size than those reported (13) for *Veratrum viride* and relatively more scalariform vessels and tracheids occurred than in powdered *V. viride* drug. None of the endodermal cells of the root showed any V-shaped lumina as reported to occur in the endodermis of some roots of *V. viride* (12).

Veratrum Seed Germination and Seedlings

The following preliminary tests were carried out in the experimental germination of *Veratrum* seeds and the results are reported together with observations on the seedlings obtained.

Fifty seeds of *Veratrum viride* obtained from wild plants growing in New Hampshire and the same number of seeds of *Veratrum album* obtained from Amsterdam, Netherlands, were planted in separate 10-in. pots containing soil consisting of a mixture of washed sharp beach sand, garden loam, and humus. The soil was pressed firmly down. The seeds were then distributed over its surface, and a fine, screened mixture of the soil was sprinkled on top to the depth of $^1/_8$ in. and pressed down. This was then covered with cheesecloth and sprinkled gently with a fine spray of water. The pots were then set out in the greenhouse and watered only from below by setting in shallow pans to which water alone was added. After about eleven months from the date of planting, 4 linear leaves made their appearance above ground. These were the primary leaves of first year seedlings of *Veratrum viride*, and indicated only 8% of germination under the conditions described. None of the *V. album* seeds germinated during the fifteen months of observation after planting.

A second series of fresh *Veratrum viride* seeds, also from wild growing New Hampshire plants, were planted under the conditions of planting and culture described for the first lot, except that the seeds were first refrigerated for two months at a temperature range of between 32 and 35° F. and subsequently dusted with Arasan by agitating them with this fungicide in a fine mesh strainer, immediately after refrigeration and just before planting in two pots which were set in a shaded spot of the greenhouse. Within a year this lot yielded 14 seedlings each with one linear leaf and a bulb-like subterranean organ. During the second year the plants were watered from above and early in the year each produced a single lanceolate secondary leaf which was followed by another leaf of the same shape.

The young seedlings were transplanted to flats containing a layer of sand below, and fertilized loam and acid woodland soil on top; they were watered and placed in the shade to be kept under observation until sufficiently developed to be planted in moist, well-drained woodland.

The results of these preliminary tests show that improved germination of *Veratrum viride* seeds may be effected by giving the seeds a period of dormancy for two months under refrigeration, then dusting them with a fungicide such as Arasan (used), Semesan, or Spergon, and keeping the pots watered from below and in shade until the secondary leaves have developed.

Under these conditions the percentage of germination was 28. Further tests are in progress.

SUMMARY AND CONCLUSIONS

1. A short taxonomic history, including synonymy, of *Veratrum Eschscholtzii* A. Gray is given.

2. New observations are reported on its distribution and environment in the Pacific Northwest.

3. A collective description is given of living and dried plants of *V. Eschscholtzii* examined by the author, and some new data are reported.

4. The differences between the overground plants of the Western North American *Veratrum Eschscholtzii* and the Eastern North American *V. viride* were found to be few and largely quantitative. Greater hairiness of the leaves is reported on plants of *V. Eschscholtzii* growing in Alaska and northern Washington than on those growing southward whose hairiness approaches the scantiness of some *V. viride* leaves. While drooping side branches are typical for the panicles of *V. Eschscholtzii* and ascending side branches for the panicles of *V. viride*, plants of both species have been found which possess both drooping and ascending side branches. The mature stamens of *V. Eschscholtzii* differed from those of *V. viride* only in the greater length of their filaments.

5. The anatomy of the rhizome and of the root of *V. Eschscholtzii* is described for the first time.

6. Under reasonably good soil conditions *V. Eschscholtzii* usually produces 10 to 15 new roots each year, whereas *V. viride* forms 20 to 35.

7. A description is given of the dried rhizome and root drug of *Veratrum Eschscholtzii* earlier reported by the author to be found in commerce

as a substitute for *Veratrum viride* and now employed in the experimental extraction of alkaloids.

8. Both the rhizomes and roots of *V. Eschcholtzii* were found to attain a greater thickness than those of *V. viride*.

9. The powdered rhizome and root material of *V. Eschscholtzii* is described for the first time. Its starch grains attain a greater diameter than those of *V. viride* and the endodermal cells of its roots are larger in size and never show V-shaped lumina like some of the latter. The scalariform tracheids and vessels were found to be more numerous in powdered *V. Eschscholtzii* than in powdered *V. viride*, and a number of them were characterized by a peculiar lobing of their walls and by occasional furcate branching.

10. The results of *Veratrum* seed germination tests under different culture conditions are reported, as well as the appearance of first- and second-year seedlings of *V. viride*.

It is shown that refrigeration of the seeds for two months, dusting with Arasan or other suitable fungicide immediately prior to planting, watering the pots or flats from below, and shade are conducive to a greater yield of seedlings.

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1953 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1953 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1953 Award, nominations must be received on or before February 1, 1953.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, *Chairman;* Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, John C. Krantz, and Frank O. Taylor.

The award is now in its fifth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Lebond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex., and was conferred during the 1952 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

Composition of Gum Turpentines of Pines

XVI. A Report on *Pinus oocarpa* and *P. pseudostrobus* var. *oaxacana* from Chiapas and *P. cooperi* from Durango*

By P. M. ILOFF, Jr., and N. T. MIROV†

The sources, collection, and determination of the chemical composition of gum turpentines from three Mexican species of pine are reported. Conventional methods of fractionation by distillation, determination of physical and chemical characteristics, and the preparation of derivatives for the purpose of identifying the several fractions obtained from each of the three turpentines were employed throughout the investigation.

PINUS OOCARPA Scheide is a widely distributed pine ranging from Guatemala to the southern part of Chihuahua, Mexico. Besides the typical form, four varieties of this pine are recognized (3). The chemistry of turpentine of the presumably typical form, *Pinus oocarpa* from near Uruapan, Michoacan, Mexico, has already been reported in THIS JOURNAL [**40**, 550(1951)]. The turpentine of this Michoacan variety was found to consist almost entirely of dextrorotatory α-pinene, specific rotation $[\alpha]_D + 47.5°$.

EXPERIMENTAL

Oleoresin used in the present study was obtained with the assistance of Ing. Marcial Zebadua near the village of Rancho Nuevo, some 40 miles southwest of San Cristobal de Las Casas, Chiapas, Mexico.[1] Local Indians call this pine chagtog. The trees were carefully identified and herbarium specimens were preserved for future references to this species.

At the end of distillation the pressure was 4 mm. and the temperature was 190°. Yield of turpentine was 22% of water- and trash-free oleoresin.

The crude turpentine had the following characteristics: d^{23}_4, 0.8562; n^{23}_D, 1.4684; and $[\alpha]^{23}_D$, + 25.4°.

A batch of 571 Gm. of the turpentine was fractionated in a jacketed and heated Vigreux column 80 cm. long, equipped with a stillhead permitting 1–10 reflux ratio. The results of the distillation are shown in Table I.

Fraction 1 and Fraction 2 were mostly *d*-α-pinene. α-Pinene was identified in Fraction 1 by preparation of the nitrolpiperidide which melted at 118–119° after 3 crystallizations from 95% ethanol. There was no depression in melting point by mixing with authentic α-pinene nitrolpiperidide derived from *Pinus echinata*.

d-, *dl*-α-Pinene nitrosochloride, m. p. 104–105°, was prepared from Fraction 3.

Fraction 3 had the properties of α-pinene mixed with limonene and Fraction 4 had properties close to those of limonene. Fraction 4 was redistilled over sodium in a 50-ml. Claissen flask at atmospheric pressure. A heart cut had the following properties: $b_{.780}$, 174–176°; n^{24}_D, 1.4714; d^{23}_4, 0.8427; $[\alpha]^{23}_D$, −83.9°. A tetrabromide was prepared which, after recrystallization twice from methanol, had a melting point of 103–104° and showed no depression with authentic limonene tetrabromide.

Fraction 5 had the properties of a mixture of sesquiterpene and limonene but was predominately limonene.

Fractions 6, 7, 8, and 9 contained sesquiterpene. Fraction 6 was redistilled over sodium through a 3½-in. Vigreux column. About three-fourths of the distillate had the following properties: $b_{.10}$, 90–95°; $n^{23.5}_D$, 1.5040; d^{24}_4, 0.9368; R_D, 64.5. The preparation of a hydrochloride was attempted, but no crystalline derivative was obtained.

Fraction 8 was redistilled over sodium through a 3½-in. Vigreux column. A heart cut (over 50%) had the following physical characteristics: $b_{.9}$, 112–113°; n^{23}_D, 1.5020; d^{23}_4, 0.9301; R_D, 64.6. A good yield of crystalline hydrochloride was prepared from oil obtained from the heart cut by saturation of an ether solution with dry HCl gas. The derivative melted at 59° after recrystallization from glacial acetic acid. There was no depression in melting point on mixing with longifolene hydrochloride from *P. pseudostrobus* turpentine. A density *vs.* refractive index diagram of the first 4 fractions was plotted and from this the percentage of limonene and α-pinene was estimated (5).

Thus, the composition of the turpentine from *P. oocarpa* from Chiapas proved to be relatively simple. It consists of about 68% *d*- and *dl*-α-pinene, 22% *l*- and *dl*-limonene; and 7% sesquiterpene, most of which is *d*-longifolene.

Pinus pseudostrobus var. oaxacana Martinez.—*Pinus pseudostrobus* is a species growing in the warmer parts of Mexico. Its range extends south to Guatemala and probably Nicaragua. In Mexico, it is represented by the typical form and by four varieties (3). A sample of oleoresin for the present study was collected from variety *oaxacana* in the State of Chiapas, in the same locality as *Pinus oocarpa* of this article. Local people call this pine *Juncia delgada* because of its sedge-like, delicate foliage.

Upon distillation of 3,107 Gm. of oleoresin under reduced pressure (3 mm. and 180° at the end) the yield of turpentine was 17% of water- and trash-free oleoresin.

* Received October 8, 1952, from the California Forest and Range Experiment Station, maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

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[1] The oleoresin collecting trip was made by N. T. Mirov in the summer of 1950 with the assistance of the associates of Subtropical Geography, University of California. Thanks are due to Dr. Carl O. Sauer of the Department of Geography of the University for his interest in the project.

The crude turpentine had the following physical characteristics: d_4^{24}, 0.792; n_D^{23}, 1.4445; $[\alpha]_D^{24}$, −10.5°. The density and refractive index of the crude turpentine were significantly lower than the values for most turpentines because of the presence of paraffin hydrocarbons.

A batch of 447 Gm. of the turpentine was fractionated in an 80-cm. heated-jacket Vigreux column in which a reflux-distillate ratio of 10–1 was maintained. The results of the distillation are shown in Table I.

A portion of Fraction 1 and Fraction 2 combined was repeatedly shaken with fresh quantities of fuming sulfuric acid until the acid remained colorless upon shaking with the hydrocarbon. The hydrocarbon was washed with concentrated sulfuric acid, then with dilute aqueous sodium bicarbonate, and finally with water. After drying over sodium sulfate, a 90.7% yield of colorless hydrocarbon was received. The distilling range at atmospheric pressure was 97 to 98.5°. The physical properties of a heart cut (73% of the distillate) are compared with values for pure heptane in Table II.

An infrared absorption spectrum of this purified heptane is tabulated in Table III for comparison with heptane from the American Petroleum Institute Project 44. A sodium chloride prism was used in a double-beam Perkin-Elmer spectrophotometer for all infrared spectra taken for this paper.

It is estimated that the turpentine of *P. pseudostrobus* contains about 21% heptane.

Fractions 3, 4, 5, and 6 contained mostly *d*- and *dl*-α-pinene with some heptane in the lower boiling fractions and limonene in the higher boiling fractions. A good yield of *dl*-α-pinene nitrosochloride was prepared from Fraction 4. After recrystallization from chloroform by addition of cold methanol, the derivative melted at 103°. No depression of melting point was observed on mixing it with α-pinene nitrosochloride from *Pinus cooperi* turpentine.

Fraction 6 was repeatedly distilled and appeared to be a mixture of α-pinene and limonene, judging from the physical constants of the fractions obtained.

TABLE II

Heptane from *P. pseudostrobus*	n-Heptane (4)	
n_D 1.3865[23]	1.38764[20]	1.38517[25]
d 0.68052[4]	0.68368[20]	0.67947[25]
B. P. 98–98.5°	98.428°	

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS OOCARPA FROM CHIAPAS, P. PSEUDOSTROBUS VAR. OAXACANA, AND P. COOPERI

Fractions	Pressure, Mm.	Boiling Range, °C.	Distillate, %	Density, d_4^{25}	Index of Refraction, n_D^{25}	Specific Rotation $[\alpha]_D^{25}$
Pinus oocarpa from Chiapas (571 Gm. Used)						
1	755	155–157	54.6	0.8523	1.4645	+43.1
2	755	157–163	21.1	0.8508	1.4659	+29.5
3	755	163–172	7.8	0.8458	1.4686	−20.7
4	755	172–175	4.6	0.8410	1.4710	−72.5
5	10	65–95	3.7	0.8712	1.4833	....
6	10	95–100	1.1	0.9368	1.5028	+18.1
7	10	100–115	0.9	....	1.5035	....
8	10	115–118	2.5	0.9279	1.5012	+48.2
9	1.5	68	0.6	0.9257	1.5017	....
Pot residue	...	.....	1.3	....	....	....
Losses	...	.....	1.8	....	....	....
Pinus pseudostrobus var. *oaxacana* (447 Gm. Used)						
				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	760	87–98	17.9	0.7140	1.3847	+0.4
2	760	98–150	7.0	....	1.4094	....
3	760	150–154	2.6	....	1.4454	....
4	760	154–158	24.0	0.8296	1.4553	+4.3
5	760	158–162	12.1	0.8377	1.4607	−3.9
6	760	162–168	9.4	0.8387	1.4640	−19.0
7	18	58–68	4.5	0.8411	1.4672	−42.5
8	18	68–70	5.0	0.8358	1.4686	−84.0
9	18	70–78	4.3	0.824	1.4645	−90.7
10	18	78–91	2.9	0.7903	1.4369	−17.0
11	18	91–101	2.9	0.8850	1.4746	−8.7
12	4.5	85–108	4.6	0.9200	1.4951	+27.3
13	4.0	150–165	0.5	0.9108	1.5075	....
Residue and loss	...	.....	2.3	....	....	....
Pinus cooperi (612 Gm. Used)						
				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	759	155–157	56.2	0.8576	1.4691	+10.0
2	759	157–158.2	22.7	0.8602	1.4718	−0.2
3	759	158.5–160	8.3	0.8617	1.4742	−8.8
4	759	160–161	7.5	0.8632	1.4769	−14.6
Pot residue	...	Above 161	4.2	....	....	....
Losses	...	.....	1.1	....	....	....

Both limonene tetrabromide and α-pinene nitrosochloride were prepared from the appropriate fractions.

l-Limonene was identified in Fraction 7 by preparation of the limonene tetrabromide. Fraction 8 was almost pure limonene and upon distillation over sodium at atmospheric pressure yielded a heart cut having the following properties: n_D^{25}, 1.4688; $b._{760}$, 175–176°; $d_4^{26.5}$, 0.8361; $[\alpha]_D^{26.5}$, −88.8°. Addition of bromine to 1 Gm. of this fraction dissolved in 1:1 ethanol-ether yielded the tetrabromide, which after two recrystallizations from hot methanol melted at 104° and showed no depression in melting point when mixed with authentic limonene tetrabromide.

TABLE III—ABSORPTION MAXIMA IN THE INFRARED REGION FOR *n*-HEPTANE AND *n*-UNDECANE[a]

Maxima in Wave Numbers			
n-Heptane, A. P. I. Infrared Spectrum #637	*n*-Heptane from *Pinus pseudostrobus*	*n*-Undecane, A. P. I. Infrared Spectrum #391	*n*-Undecane from *Pinus pseudostrobus*
723	723	721	719
739	739	761	761
774	773	780	778
833	832	876	824
864	867	843	..
876	877	890	888
901	902	906	904
931	933	948	945
961	961	960	..
990	..	1110	1002
1022	1022	1076	1076
1076	1077	1094	1095
1039	1140	1135	1134
1209	1210	1180	1179
1235	1238	1206	1206
1280	1282	1241	1243
1302	1303	1270	1272
1342	1344	1303	1303
1380	1382	1342	1343
		1354	1353
		1379	1379
		1467	1465

[a] Rossini, Frederick D., *et al.*, "Catalog of Selected Infrared Absorption Spectrograms," American Petroleum Institute Research Project 44, National Bureau of Standards, Washington, D. C., 1946–1951.

Fraction 9 contained *l*- and *dl*-limonene with the density and refractive index values somewhat low because of the presence of a small amount of paraffin hydrocarbon.

Fraction 10, consisting of 13.1 Gm. of material, was treated with fuming sulfuric acid in the manner in which a portion of fractions 1 and 2 were treated. The yield of hydrocarbon was 44%; it was distilled at atmospheric pressure over sodium metal. The properties of the distillate compared with the properties of pure *n*-undecane are shown in Table IV.

The infrared absorption spectrum is compared with that for *n*-undecane in Table III.

It is concluded that the turpentine of *P. pseudostrobus* var. *oaxacana* contains about 1.3% of the normal hydrocarbon undecane.

Fractions 11 and 12 have physical properties indicating that they contain a sesquiterpene. Fraction 12 was redistilled in a Claisen flask over sodium, yielding 2 fractions which were each redistilled giving heart cuts having the following characteristics: Cut A—$b._{4}$, 91–98°; $n_D^{22.3}$, 1.4993; $d_4^{19.5}$, 0.9274; Cut B—$b._{11}$, 60–70°; $n_D^{19.5}$, 1.5012; $d_4^{19.5}$, 0.9310. Hydrochlorides of both Cut A and Cut B were prepared. Both hydrochlorides melted at 59 to 59.5° and showed no depression of melting point upon admixture of a sample of longifolene hydrochloride from *P. montezumae* from Chiapas.

A hydrochloride was prepared from Fraction 13 by saturation with dry HCl gas in ether. After two recrystallizations from glacial acetic acid, the hydrochloride melted at 119–120° and showed over 13° depression in melting point with cadinene hydrochloride. There was not sufficient material for analysis.

In summary, the gum turpentine of *Pinus pseudostrobus* var. *oaxacana* contained 21% heptane; 51% *d*- and *dl*-α-pinene; 15–16% *l*- and *dl*-limonene; 1.3% *n*-undecane; and 7.5% *d*-longifolene.

Pinus cooperi Blanco.—*Pinus cooperi* Blanco is a newly discovered species of pine. It was originally described by Sr. Ing. Cenobio E. Blanco in 1940 as *Pinus lutea*, probably because it is commonly known as pino amarillo, i. e., yellow pine. As the name *Pinus lutea* had been given to loblolly pine in 1788, Blanco decided, on the suggestion of Dr. Elbert L. Little, Jr., to change its name. Accordingly, Blanco in 1949 changed the name from *P. lutea* to *P. cooperi* (1). Taxonomically this pine is in the group of pines that Shaw lumps together under the name of *Pinus montezumae*. Martinez places it between *Pinus rudis* and *P. michoacana*. It is hoped that the chemical analysis of its turpentine may help in establishing the relationship of this pine to the other pines. *Pinus cooperi* grows in the mountain ranges of Durango, Mexico. At El Salto, Dgo., it occurs at elevations of 8,200 to 9,000 feet. In the same locality also occurs a variety of this pine (*P. cooperi*, var. *ornelasi*) characterized by coarser bark, longer needles, larger cones, and whiter wood than *P. cooperi* proper. Local people also distinguish these two pines and call the *ornelasi* variety not pino amarillo, but albcarrote. Turpentine of this variety is under investigation.

TABLE IV

	n-Undecane from *P. pseudostrobus*	*n*-Undecane (2)
n_D	$1.4165^{22.3}$	1.41902^{20}
d	0.7374_4^{24}	0.7404_2^{20}
$b._{760}$	196–197°	195.8°

Oleoresin of *Pinus cooperi* was collected at El Salto, Durango, Mexico, under supervision of Sr. C. E. Blanco, the authenticity of the pine thus being assured. Turpentine was distilled as usual under reduced pressure. At the end of distillation, the pressure was 4 mm. and the temperature was 180°. Yield of turpentine was 25%.

The crude turpentine of *P. cooperi* had the following physical characteristics: n_D^{23}, 1.4705; d_4^{23}, 0.8580; $[\alpha]_D^{23}$, +2.9°.

A batch of 612 Gm. of the turpentine was distilled at 759 mm. pressure in an 80-cm. Vigreux column equipped with heated jacket. The results of the distillation are shown in Table I.

After converting the densities and refractive indexes of the first four fractions to values for 25°,

a curve was plotted with densities as ordinates and refractive indices as abscissae. The conversion factors used were 0.0008 per degree centigrade for density and 0.00045 per degree centigrade for refractive index. Upon comparing the values for the first four fractions with the values for α- and β-pinene it was concluded that α-pinene was present in these fractions to the extent of 56% of the original turpentine, and that β-pinene was present to the extent of 39% (5).

Fraction 1 gave a good yield of α-pinene nitrosochloride, m. p. 103°. α-Pinene was identified in Fraction 3 by preparation of the nitrosochloride having a melting point of 106 to 106.5° after recrystallization from chloroform by addition of cold methanol.

Judging from the physical constants, Fractions 3 and 4 would be expected to contain a high proportion of β-pinene. Sodium nopinate and nopinic acid were prepared from Fractions 4 by alkaline permanganate oxidation (for methods, see previous articles of this series in THIS JOURNAL). The nopinic acid was recrystallized from hot water and had a melting point of 125.5 to 126°. A melting point of its mixture with authentic nopinic acid showed no depression.

Preparing a hydrochloride of the pot residue was attempted but was unsuccessful. The pot residue was distilled at atmospheric pressure over a range of 167–220°. The high boiling fraction (179–220°) was redistilled over sodium at atmospheric pressure; decomposition occurred. The refractive index of the highest boiling cut (183–193°) was n_D^{23}, 1.4767, which indicated that it did not contain very much sesquiterpene. The residue was not investigated further.

To sum up, the turpentine of *Pinus cooperi* contains 56% *d*- and *dl*-α-pinene; 39–40% *l*-β-pinene; and 3–4% unidentified higher boiling material.

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(3) Martinez, M., "Los Pinos Mexicanos," 2nd ed., Ediciones Botas, Mexico, 1948, pp. 300–322 (*Pinus oocarpa*); 184–209 (*Pinus pseudostrobus*).
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Fellowship for Graduate Study of Food and Drug Law

The New York University School of Law has established a national center of graduate instruction in the food and drug law, conducted in association with The Food Law Institute. The program for it includes four courses in this law in general and in the major Federal Food, Drug, and Cosmetic Act in particular, and it provides for basic research studies thereof. Mr. Charles Wesley Dunn has professorial charge, and Messrs. William W. Goodrich and Vincent A. Kleinfeld, food and drug attorneys of the Federal Government, present systematic lectures.

The students are practicing lawyers, who register for this program, and five selected fellows. The latter are recent law school graduates throughout the country, who are chosen for their high academic and personal qualifications; they are thus trained to become expert practitioners or teachers of this law. Each fellow is a candidate for a Master of Laws degree, and most fellows then proceed to secure a Doctor of Juridical Science degree. This year the fellows are graduates from the law schools of the following Universities: Harvard, Stanford, Virginia, Washington, and New York. Most of the graduated fellows now teach or lecture on the food and drug law in different University Law Schools.

One fellowship in this program is underwritten largely by the American Pharmaceutical Manufacturers' Association. Applications for this fellowship for 1953–1954 are now sought for a selection early in 1953. Preferred applicants are those who have received degrees in both law and pharmacy. The stipend ranges up to $3,000 and is determined by the circumstances. Address all applications to The Food Law Institute, Suite 1004, 608 Fifth Avenue, New York 20, N. Y. Each applicant should make a complete statement of his background and cite reference names.

Amino Acid Derivatives of Aminosalicylic Acids*,†

By WILLIAM O. FOYE and ROBERT L. HULL‡

A series of amide derivatives of 3-, 4-, and 5-aminosalicylic acids with α-amino acids has been prepared by a method which involves amide formation between the carboxyl group of the aminosalicylic acid and the amino group of the amino acid. The stability as well as the ease of formation of the compounds was increased by utilization of the O-benzyl ethers of the nitrosalicylic acids and ester hydrochlorides of the amino acids.

A VARIETY of simple derivatives and analogs of *p*-aminosalicylic acid (PAS) involving either the carboxyl, amino, or hydroxyl group have been prepared and tested for tuberculostatic activity following the announcement by Lehmann (1) of the antitubercular effect of PAS. The results of the tuberculostatic activities of more than 100 of these compounds led Hirt and Hurni (2) to conclude that while the majority did not exceed PAS in antitubercular activity, it was possible that some derivative was formed *in vivo* which greatly extended the activity of PAS beyond that which could reasonably be expected. If this is true, conjugates of PAS with naturally occurring compounds such as amino acids, peptides, sugars, etc., might be expected to show activity comparable to that of PAS itself. Accordingly, the preparation of a series of amino acid derivatives of 4-aminosalicylic acid was undertaken to compare their antitubercular effects.

It was decided to make amides of the carboxyl group of PAS, since Lehmann (3) had already reported that, in general, substitution on the carboxyl group changed the activity only slightly. Since this work was started, Doub, *et al.* (4), have indicated that with the exception of esterification, modification of the carboxyl group generally reduces activity. They did prepare the glycine amide of PAS, however, and found remarkably high activity which was abolished in the presence of serum. The compounds reported in the present paper contain esterified amino acid groups which, it is hoped, will hinder serum reactions. Amino acid amides of the 3- and 5-aminosalicylic acids were also prepared, since derivatives of these acids have been found to possess some degree of activity (1).

The nitrosalicylic acids were used as starting materials for the syntheses of the amino acid amides in order to avoid side reactions of the amino group of the aminosalicylic acids or attempted preferential hydrolyses of the variously blocked amino groups. The 3- and 5-nitrosalicylic acids are commercially available; the 4-nitro isomer was synthesized by modifying the procedure of Borsche (5). Attempts to prepare the acid chlorides of the nitrosalicylic acids included the reaction of the sodium salts with thionyl chloride, acetyl chloride, and phosphorous oxychloride, but with only one amino acid (*dl*-alanine) was acid chloride formation indicated by subsequent amide formation (13 per cent yield, using thionyl chloride). Blocking the phenolic group of the nitrosalicylic acids with the acetyl group was equally unsuccessful, due to hydrolysis in the aqueous solutions necessary for dissolving the amino acids. Doub (4), however, has reported the preparation of 2-acetoxy-4-nitrobenzoyl chloride in acetyl chloride solution, and was successful in obtaining amides from it. The method of Clinton, *et al.* (6), for making aminosalicylic acid esters utilizing the labile O-benzyl group which is readily removed by catalytic hydrogenation (a procedure also necessary for final reduction of the nitro groups) was found applicable. Amide formation between the benzyloxy nitrosalicylic chlorides and amino acids then proceeded with moderate yields.

The acid chlorides were prepared in benzene solution, and in order to avoid aqueous media and provide more stable products, ester hydrochlorides of the amino acids were used directly in the organic solvent. A critical temperature of 40–50° was found for amide formation in all the reactions observed, and variations above or below resulted in negligible yields. It was also found that the ester group of the amides could be preferentially hydrolyzed to the free acid amides without affecting the rest of the molecule, illustrated by the formation of 2-benzyloxy-5-nitrobenzoylglycine. Ordinarily, however, the ester group was retained to give greater stability, particularly necessary during the formation of the amine hydrochlorides of the final products.

Amide formation of 3-nitrosalicylic acid by this procedure gave exceedingly poor yields, prob-

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† Abstracted from a thesis presented to the Graduate School of the University of Wisconsin by Robert L. Hull in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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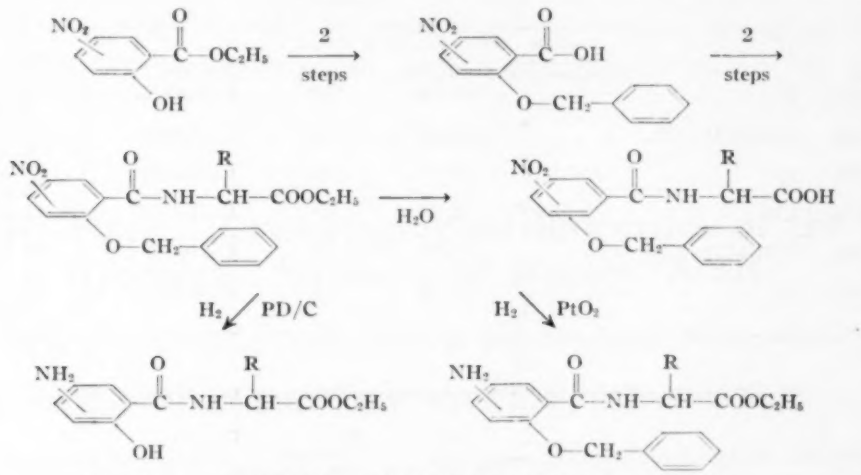
ably because of the sterically hindered phenolic group. 2-Benzyloxy-3-nitrobenzoic acid, for instance, underwent considerable decomposition when dried at 100°. For this reason, only one 3-nitrosalicylamide was converted to the final aminosalicylamide.

The nitro amides were readily hydrogenated over palladium-on-charcoal; the calculated amount of hydrogen was taken up to reduce the nitro group and cleave the O-benzyl ether. The resulting aminophenol solutions underwent immediate darkening, probably due to air oxidation, as indicated by a clearing of the color on rehydrogenation. Isolation of solid, crystalline products was unsuccessful, however, although the solutions gave positive tests for primary aromatic amine and phenol groups. The benzyl group was then retained in the products by hydrogenation with platinum oxide, which selectively reduced the nitro group. Stable, crystalline solids were isolated on formation of the amine hydrochlorides, although in several instances the free base was more readily obtained. The general reaction is illustrated in Eq. 1.



Equation 1.—Synthesis of amino acid amides of aminosalicylic acids.

EXPERIMENTAL

4-Nitrosalicylic Acid.—This acid was prepared according to the method of Borsche (5), from 2,4-dinitrophenylacetic ester (7) by ring closure to 3-carbomethoxy-6-nitrobenzisoxazole and subsequent hydrolysis. It was found more effective, however, first to hydrolyze the isoxazole to 4-nitrosalicylonitrile, m. p. 160–161°, by refluxing in 5 *N* hydrochloric acid for four hours, and further hydrolyze the isolated nitrile to the acid by refluxing in 70% sulfuric acid for twenty minutes. Over-all yields of 28–30% (from phenylacetic acid) were obtained of cream-colored needles melting at 233–235°.[1]

[1] All melting points are corrected.

5-Nitrosalicyl-*dl*-alanine.—To a suspension of 10.0 Gm. (0.049 mole) of dry sodium 5-nitrosalicylate in 75 ml. of dry benzene was added slowly 16.5 Gm. (0.139 mole) of thionyl chloride. The mixture was stirred for thirty minutes at room temperature and was then evaporated to dryness under reduced pressure. To the colorless residue was added a solution of 4.4 Gm. (0.049 mole) of *dl*-alanine and 6.0 Gm. (0.150 mole) of sodium hydroxide in 125 ml. of water. After being shaken for fifteen minutes, the reaction mixture was heated on a steam bath for thirty minutes and filtered, and the filtrate was acidified. The white precipitate of 5-nitrosalicylic acid (6.9 Gm.) was removed, and the filtrate was reduced to one-half its volume and cooled. The resulting 5-nitrosalicyl-*dl*-alanine, after two recrystallizations from aqueous alcohol, weighed 1.5 Gm. (13%) and melted at 184–186°.

Anal.—Calcd. for $C_{10}H_{10}O_6N_2$: C, 47.25; H, 3.97. Found: C, 47.06; H, 3.78.[2]

Ethyl Nitrosalicylates.—Ethyl 5-nitrosalicylate (m. p. 95–96°) was prepared in 85% yield by the method of Hirsch (8). Ethyl 4-nitrosalicylate (m. p. 87–88°) was prepared in 74% yield according to Borsche (5), and the 3-nitrosalicylate (m. p. 43.5 to 44.5°) in 89% yield by Zacharias' method (9).

Benzyloxynitrobenzoic Acids.—The procedure of Clinton, *et al.* (6), for 2-benzyloxy-4-nitrobenzoic acid was employed for the preparation of these compounds from the ethyl nitrosalicylates. It was found more advantageous to isolate the hydrolyzed benzyloxy acids, however, by evaporating the aqueous alcoholic solutions to one-half their volume and pouring the remainder into a liter or more of water before acidification. 2-Benzyloxy-4-nitrobenzoic acid (m. p. 171.5 to 173°) was obtained in 88% yield, and 2-benzyloxy-5-nitrobenzoic acid (m. p. 169–170°) was obtained in 50% yield.

Anal.—Calcd. for $C_{14}H_{11}O_5N$: C, 61.54; H, 4.06. Found: C, 61.76; H, 3.95.

In the preparation of 2-benzyloxy-3-nitrobenzoic acid, it was found necessary to carry on the benzyl-

[2] The analyses were carried out by the Clark Microanalytical Laboratory, Urbana, Ill.

ation for nineteen hours. After two recrystallizations from isopropyl alcohol, a 35% yield was obtained of product which melted at 128–129°.

Anal.—Calcd. for $C_{14}H_{11}O_6N$: C, 61.54; H, 4.06. Found: C, 62.04; H, 4.26.

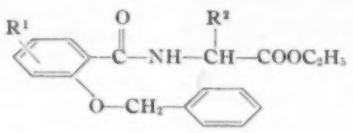
Amino Acid Ester Hydrochlorides.—The method of Curtius and Koch (10), involving the saturation of an ethanolic suspension of the amino acid with dry hydrogen chloride, was employed in each case. Ethyl glycine hydrochloride (m. p. 142–143°) was prepared in 91% yield, ethyl *dl*-alanine hydrochloride (m. p. 70–74°) in 83% yield, and ethyl *dl*-leucine hydrochloride (m. p. 110–112°) in 85% yield.

Ethyl Amino Acid Amides of the Benzyloxynitrobenzoic Acids.—To a stirred mixture of 10.0 Gm. (0.037 mole) of benzyloxynitrobenzoic acid, 9.25 Gm. (0.117 mole) of pure, dry pyridine, and 150 ml. of dry benzene, 4.52 Gm. (0.038 mole) of thionyl chloride was added dropwise during a period of five minutes at room temperature. The mixture was stirred and refluxed for twenty minutes, and cooled to 40°; an equivalent (0.037 mole) of amino acid ester hydrochloride was then added slowly with stirring. After stirring for one-half to six hours at 40–50°, the warm mixture was filtered to remove pyridine hydrochloride. The filtrate was washed with 5% hydrochloric acid, 5% sodium carbonate, and water; after drying over Drierite, the benzene solution was either evaporated to dryness or diluted with an equal volume of Skellysolve B. The resulting solid or oil was recrystallized from alcohol or aqueous alcohol until pure. The physical constants and yields of the products are in Table I.

In the preparation of ethyl 2-benzyloxy-3-nitrobenzoylglycine, triethylamine was required as the base, and the twenty-minute period of refluxing for the acid chloride formation had to be omitted. It was also found impossible to purify the ethyl 2-benzyloxy-5-nitrobenzoylalanine sufficiently to obtain a correct carbon-hydrogen analysis.

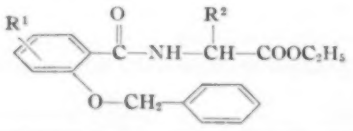
2-Benzyloxy-5-nitrobenzoylglycine.—A suspension of 3.9 Gm. (0.011 mole) of ethyl 2-benzyloxy-5-

TABLE I.—ETHYL AMINO ACID AMIDES OF BENZYLOXYNITROBENZOIC ACIDS



R¹	R²	Reaction Time (Amide Formation), Hr.	M. P., °C.	Yield, %	Formula	Analyses, % Calcd.	Found
5-NO_2	H	1/2	147–148	39	$C_{15}H_{13}O_6N_2$	C: 60.33	59.95
						H: 5.06	4.78
5-NO_2	$(CH_3)_2CH—CH_2$	2	126–127.5	24	$C_{22}H_{26}O_6N_2$	C: 63.75	64.56
						H: 6.32	6.40
4-NO_2	H	1/2	133–134	27	$C_{15}H_{13}O_6N_2$	C: 60.33	59.97
						H: 5.06	5.03
4-NO_2	CH_3	1/2 (40–50°) 14 (R.T.)	72–74	18	$C_{19}H_{20}O_6N_2$	C: 61.28	62.81
						H: 5.41	5.25
4-NO_2	$(CH_3)_2CH—CH_2$	6	75–77	22	$C_{22}H_{26}O_6N_2$	C: 63.75	64.67
						H: 6.32	6.51
3-NO_2	H	1/2	63–64	3	$C_{15}H_{13}O_6N_2$	C: 60.33	60.56
						H: 5.06	5.06

TABLE II.—ETHYL AMINO ACID AMIDES OF BENZYLOXYAMINOBENZOIC ACIDS



R¹	R²	Isolate	M. P., °C.	Yield, %	Formula	Analyses, % Calcd.	Found
5-NH_2	H	HCl	206–207	71	$C_{15}H_{21}O_4N_2Cl$	C: 59.26	59.27
						H: 5.80	5.93
5-NH_2	$(CH_3)_2CH—CH_2$	HCl	163–165 (dec.)	50	$C_{22}H_{29}O_4N_2Cl$	C: 62.77	62.02
						H: 6.94	6.15
4-NH_2	H	HCl	162–164	60	$C_{15}H_{21}O_4N_2Cl$	C: 59.26	59.15
						H: 5.80	5.87
4-NH_2	CH_3	Base	124–125	65	$C_{19}H_{22}O_4N_2$	C: 66.65	68.71
						H: 6.48	6.55
4-NH_2	$(CH_3)_2CH—CH_2$	Base	119–120	97	$C_{22}H_{28}O_4N_2$	C: 68.72	67.53
						H: 7.34	7.27
3-NH_2	H	HCl	102–103	29	$C_{15}H_{21}O_4N_2Cl$	C: 59.26	59.06
						H: 5.80	5.92

nitrobenzoylglycine and 33 ml. (0.033 mole) of 1 *N* sodium hydroxide solution was stirred and heated gently until practically all the solid was dissolved. After filtration, the solution was cooled and acidified with concentrated hydrochloric acid. The precipitate was collected and recrystallized twice from ethanol, giving 3.0 Gm. (83%) of colorless crystals, m. p. 190–191°.

Anal.—Calcd. for $C_{16}H_{14}O_6N_2$: C, 58.18; H, 4.27. Found: C, 57.96; H, 4.26.

Reduction of the Benzyloxynitrosalicylamides.—A mixture of the ethyl benzyloxynitrobenzoylamino acid amide (0.001 to 0.01 mole), Adams platinum oxide catalyst (0.1 Gm.), and absolute alcohol (100–150 ml.) was shaken with hydrogen at room temperature and 40 p. s. i. The theoretical amount of hydrogen was taken up in fifteen minutes or less, the catalyst was removed by filtration, and the filtrate (or a partially concentrated liquor) was saturated with dry hydrogen chloride. The cooled solution was treated with dry ether (100–400 ml.), and after being chilled, was filtered to give fine crystals of the amine hydrochloride. The products were recrystallized from alcohol-ether solutions.

In cases where it was more convenient to isolate the free base, the solution (after removal of the catalyst) was heated to boiling and treated with water to the point of cloudiness. After chilling, the precipitate was removed and recrystallized from aqueous alcohol. The physical constants and yields of the ethyl amino acid amides of the benzyloxyaminosalicylic acids are listed in Table II.

SUMMARY

1. A general method for the preparation of amino acid amides of the aminosalicylic acids has been developed.

2. A selected series of glycine, alanine, and leucine ester amides of 3-, 4-, and 5-aminosalicylic acids has been prepared for tuberculostatic testing.

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The Optical Crystallographic Properties of the Hydrolytic Products of Nucleic Acids

II. The Properties of Some Natural and Synthetic Nucleosides*,†

By JOHN A. BILES,‡ NORMAN F. WITT, and CHARLES F. POE§

The optical crystallographic properties of eleven nucleosides and three intermediates are reported. These data afford a rapid and specific method for identification.

INVESTIGATIONS have shown that the nucleus of every cell consists of nucleoproteins. Some of the most important materials containing nucleoproteins are the genes, chromosomes, certain viruses, bacteriophages, and antigens. The hydrolysis products of nucleic acids, i. e., nucleotides, nucleosides, and purine and pyrimidine bases, are important since they are found in part or in whole in many enzymes and vitamins (1).

The identification of the nucleosides usually involves the hydrolysis and identification of the sugar and purine or pyrimidine bases. The identification of these hydrolysis products of nucleic acids in mixtures is a difficult procedure. The use of optical crystallographic properties of the nucleosides for purposes of identification has not been reported in the literature. The authors (2) have reported the optical crystallographic properties of some purines, pyrimidines, and related compounds.

* Received April 11, 1952, from the College of Pharmacy, University of Colorado, Boulder.
† The research reported herein was completed under Atomic Energy Commission Contract No. A. T. (29-1)787.
‡ Atomic Energy Commission Predoctoral Fellow, 1949.
§ For reprints, address the Dean, College of Pharmacy, University of Colorado, Boulder, Colo.

TABLE I

Compound	Habit	System	Common Orientation	Extinction	Elongation	Dispersion	Axial Angle	Sign	Refractive Indexes α	β	γ
1-(*d*-2,3,4-Triacetylxylosyl)-1,2-dihydro-2-keto-4-ethoxypyrimidine	Columnar	Monoclinic	Bxa, optic axis	$Y \parallel b$, $Z \wedge c$, 25°	Bxa, −; Bxo, +	Axial, r > v	73°	−	1.446	1.526	1.577
1-(*d*-2,3,4,6-Tetraacetylglucosyl)-1,2-dihydro-2-keto-4-ethoxypyrimidine	Columnar	Monoclinic	Optic axis	$Y \parallel b$, $X \wedge c$, 39°	Bxa, −; Bxo, +	Axial, r > v	53°	−	1.472	1.527	1.542
1-(*d*-2,3,4-Triacetylarabinosyl)-1,2-dihydro-2-keto-4-ethoxy-5-methylpyrimidine	Columnar	Monoclinic	Bxo	$Y \parallel b$, $Y \wedge c$, 28°	Bxo, +	Strong crossed, v > r	46°	−	1.507[a]	1.528	1.532
1-*d*-Xylosyluracil	Columnar	Orthorhombic	Bxa and optic axis	$Y \parallel c$, $Z \parallel b$, $X \parallel a$ [b]	Bxa, +; Bxo, −	None observed	53°	+	1.531	1.561	1.705
1-*d*-Glucosyluracil	Lamellar	Hexagonal	Optic axis	Parallel and complete	Flash figure, +	None	0°	+	1.594 (ε)	1.566 (ω)	...
1-*d*-Arabinosylthymine	Columnar	Orthorhombic	Off-centered Bxa	$X \parallel c$, $Z \parallel a$, $Y \parallel b$	(−)	Axial, r > v	29°	+	1.561	1.568	1.687
1-*dl*-Arabinosylthymine	Columnar	Monoclinic	Optic normal	$X \parallel b$, $Y \wedge c$, 38°	(−)	Slight axial r > v	87°	+	1.483	1.586	1.708
1-*d*-Galactosylcytosine HCl	Tabular	Orthorhombic[c]	Bxa	$X \parallel c$, $Y \parallel a$, $Z \parallel b$	(+)	Axial, v > r	88°	−	1.489	1.591	1.672
Cytidine	Columnar	Monoclinic	Off-centered Bxa	$X \parallel b$, $Y \wedge c$, 38°	Bxa, −; Bxo, ±	Slight axial r > v	53°	+	1.578	1.600	1.697
1-*d*-Glucosylcytosine HCl	Tabular	Monoclinic	Bxo	$X \parallel b$, $Y \wedge c$, 14°	(−)	Axial, v > r	82°	−	1.510	1.614	1.710
Uridine	Acicular	Monoclinic	Optic normal	$Y \parallel b$, $X \wedge c$, 43°	Bxo, −	Strong inclined v > r	34°	−	1.539	1.646	1.657
1-*d*-Galactosyluracil	Tabular	Monoclinic	Off-centered Bxa	$Z \parallel b$, $X \wedge c$, 44°	Bxa, +; Bxo, ±	Horizontal, v > r	28°	−	1.486	1.650	1.662
Guanosine	Lamellar	Monoclinic	Optic normal	$Z \parallel b$, $X \wedge c$, 14°	Bxo, −	None observed	47°	−	1.484	1.664[d]	1.708
Adenosine	Columnar	Monoclinic	Off-centered Bxa	$Z \parallel b$, $X \wedge c$, 37°	Bxo, −; Bxa, ±; optic normal, −	Axial v > r	32°	−	1.502	1.699	1.719

[a] Crystals dissolve slowly in oils. [b] Possibly $X \parallel b$, $Z \parallel a$. [c] Pseudo-hexagonal. [d] Difficult to determine accurately as crystals are extremely thin.

EXPERIMENTAL

The compounds reported in the present communication were obtained from the University of Colorado Department of Chemistry. Nitrogen analyses had been determined before the compounds were received. The synthetic nucleosides exist as pyranosides. All of the compounds were recrystallized from water or ethyl alcohol.

A Leitz petrographic microscope Model No. 3 CM was employed, and light was obtained from a fluorescent lamp equipped with a fifteen watt G. E Mazda fluorescent bulb, 3500° white.

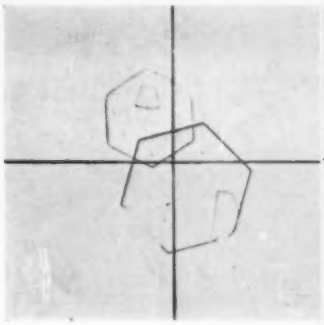
When possible, centered interference figures were employed as a guide in the determination of the principal indexes of refraction. The immersion media described by Winchell (3) were used.

The temperature at which the indexes were determined was 25° ± 1°; the accuracy of the indexes reported is ±0.002. The method of reporting elongation refers to the interference figure observed. No pleochroism was observed in any of the compounds reported. The optical angle, 2V, was calculated from the three principal indexes of refraction, by using the more exact formula suggested by Larsen and Berman (4).

Table I lists the more important optical crystallographic data of the compounds, photomicrographs of which are shown in the figures.

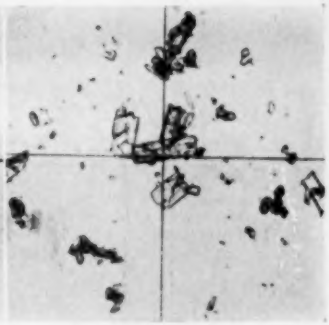
DISCUSSION

The optical crystallographic properties provided a rapid

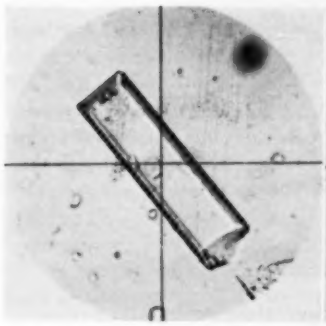


1-*d*-Glucosyluracil (260 ×).

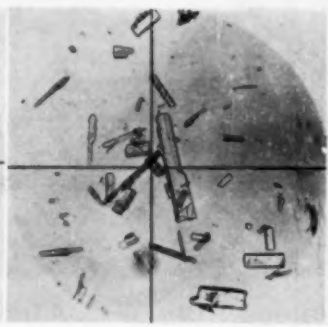
1-*d*-Galactosylcytosine hydrochloride (260 ×).



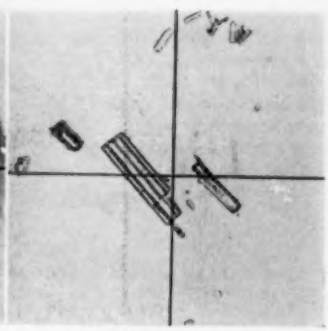
1-*d*-Glucosylcytosine hydrochloride (65 ×).



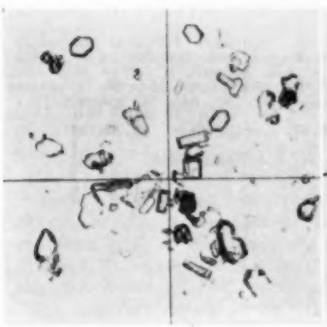
1-(*d*-2,3,4-Triacetylarabinosyl)-1, 2-dihydro-2-keto-4-ethoxy-5-methylpyrimidine (65 ×).



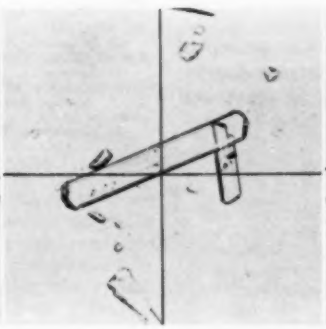
1-*dl*-Arabinosylthymine (65 ×).



1-*d*-Arabinosylthymine (260 ×).



1-*d*-Galactosyluracil (260 ×).

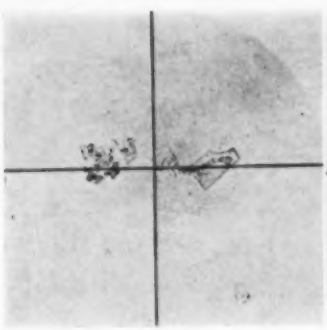


1-*d*-Xylosyluracil (260 ×).

Adenosine (260 ×).

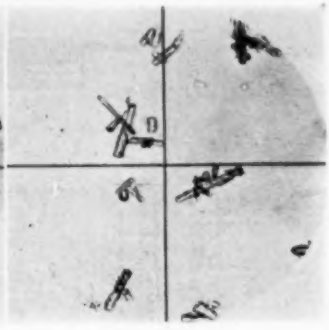
means of identifying the nucleosides studied, since well-defined interference figures were obtained with the majority of the compounds. The presence of the furanose or pyranose ring greatly reduced the birefringence found in the purine and pyrimidine bases, and the presence of acetyl groups caused a further reduction of birefringence. This condition indicates a greater random orientation than was found previously with many of the purines and pyrimidine bases.

Adenosine and guanosine showed a rather high birefringence, a condition which might be used to

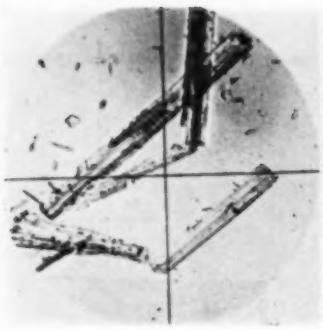


Guanosine (260 ×).

Uridine (260 ×).



Cytidine (260 ×).



1-(*d*-2,3,4-Triacetylxylosyl)-1,2-dihydro-2-keto-4-ethoxypyrimidine (65 ×).

1-(*d*-2,3,4,6-Tetraacetylglucosyl)-1,2-dihydro-2-keto-4-ethoxypyrimidine (65 ×).

substantiate the claim of Hendricks (5) that the ribofuranose ring is approximately parallel to the purine ring.

SUMMARY

1. Optical-crystallographic data are presented for eleven nucleosides and three intermediates.

2. The data obtained afford a rapid and specific method of identification.

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Dermatitis from Celery*,†

By JOSEPH F. PALUMBO and E. V. LYNN‡

In an investigation into the cause of dermatitis from celery, it has been found that the responsible agent lies in the volatile oil, which is present to the extent of about 0.005% of the fresh overground portion. Long range experiments are now under way to determine if workers in the field can be desensitized by injections of the diluted oil. During the course of the examination, the marketable portions have been submitted to proximate analysis and fractionation. Mannitol was found in abundance and some of the properties of the oil have been determined.

DERMATITIS caused by Pascal green summer celery has become a serious economic problem in Massachusetts. Celery has been cultivated in this state since the latter part of the 19th century with no record, until recently, of any untoward effects. A green summer Pascal variety was introduced here in 1935; because of its superior texture and flavor its culture spread, so that since 1939 this variety has been grown almost exclusively. Since 1939, however, large numbers of farm workers have been affected by a dermatitis, which at times has become incapacitating. Because of this condition the problems of determining the cause and the treatment of the dermatitis have become important.

The history of dermatitis from celery dates back to 1926, when it was first reported to occur on the hands of a kitchen gardener in France (1). Several other reports have been made since then, but it was not until 1948 that an attempt was made to determine the causative agent of this condition (2).

Because no conclusive scientific evidence had been given previously concerning the ingredient responsible for the dermatitis, the writers decided to make a study of the problem with the isolation of the injurious ingredients as the chief objective.

EXPERIMENTAL

Proximate Analysis.—As a preliminary to determining the causative agent of the dermatitis, a proximate analysis was made of the above-ground portions. Since there was some doubt whether the omnipresent rot is partly responsible, the examination included rotted portions as well as healthy leaves and stalks. Samples were collected during the height of the growing season and also from plants grown in a hothouse. In addition, collections were made of field strippings, which were outer portions discarded from the bunch at the field in preparation for market. Naturally these field strippings contained a high percentage of rotted material.

Weighed samples of the coarsely ground, unrotted plants were dried in the air at room temperature. The average loss of weight of three such samples on drying was 94%.

Accurately weighed samples both of field strippings and of hothouse-grown Pascal celery were burned at a dull red heat until the ash was free from carbon. Any persistent free carbon was eliminated, the percentage of acid-insoluble ash was calculated, and crude fiber determinations were made; pharmacopeial methods were used in each case. Each of the values shown in Table I is an average of three separate assays of air-dried samples.

TABLE I

	Field Strippings	Hothouse Plants
Ash, %	21.5	17.6
Acid-insoluble residue, %	3.97	1.02
Crude fiber, %	61.50	71.54

Extracts were made with ether and with alcohol on fresh and dried materials, the samples being macerated with the solvent for one week. The solution was separated by filtration and the solvent was evaporated to dryness under reduced pressure. The results shown in Table II were obtained as percentage values.

TABLE II

	Ether, %	Alcohol, %
Dried stalks	0.33	5.25
Dried leaves	1.98	15.66
Dried above-ground plant	1.20	8.45
Fresh plant	0.13	2.76
Fresh rotted field strippings	0.26	2.14

Alcoholic Fraction.—An alcoholic extraction of rotted field strippings, representing approximately 30 Gm. of a resinous material obtained from 10 Kg. of sample, was distilled with steam. The amount of volatile substance was very small and was discarded. The residual resin was dried and extracted with several solvents, but gave no crystalline

* Received August 22, 1952, from the Massachusetts College of Pharmacy, Boston.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.
† Abstracted from a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by Joseph F. Palumbo in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
‡ Research Professor of Chemistry, Massachusetts College of Pharmacy.

TABLE III

Source	Sp. Gr. at 20°	Refractive Index at 20°	Color
Air-dried rotted material	0.937	1.4888	Dark brownish-black
Air-dried hot-house celery	0.942	1.4891	Orange-yellow
Air-dried unrotted celery	...	1.5000	Yellow
Undried rotted material	...	1.5050	Dark brownish-black
Air-dried field strippings	0.977	....	Dark brownish-black

products. The residual aqueous solution was concentrated to dryness *in vacuo* and extracted with alcohol. The alcoholic solution deposited white crystals upon standing. The product was recrystallized from hot alcohol and allowed to dry in a desiccator for one week. The crystals had a melting point of 166°, the same as that of mannitol. A hexa-acetate derivative, prepared in the usual way, had a melting point of 118°. Reports in the literature indicate that the hexa-acetate derivative of mannitol melts at 119°. The hexa-acetate derivative of a known sample of mannitol when mixed with one of the sample did not change the melting point more than 2°. As far back as 1894 mannitol was reported to be present in celery but no proof was given (3). No work has been done to show if mannitol plays a part in the dermatitis.

The Volatile Oil.—Reports in the literature indicated that the dermatitis was caused by volatile oil in the plant. Therefore, the more difficult preparation of the volatile oil was undertaken to test conclusively whether this oil could cause the condition in sensitive individuals.

The volatile oil content of samples of the unground, fresh, above-ground portions of celery plants was determined by the pharmacopeial method. The oil is known to be in various parts of the stalk and in the lamina of the leaf, but only an insignificant yield of oil was obtained.

Volatile oil content was determined in a sample of coarsely ground, air-dried material by the pharmacopeial method; yield, 0.15%. This figure approximates that given in the Schimmel & Co. report of 1895 (4).

Since the fresh material yielded too small a quantity of oil for convenient handling, it was necessary to dry about 10 Kg. for the preparation of 1 cc. of the oil.

Table III shows the specific gravity and refractive index of each of several samples of oils. Specific gravity was determined using a modified Sprengel tube.

Patch Tests.—The alcoholic and ethereal extracts of the fresh, the air-dried, and the rotted material described previously, were used in patch tests together with the pure oil. These tests were performed, with the aid of Dr. F. M. Rackemann of the Massachusetts General Hospital, on six celery workers who had previously been afflicted with the dermatitis.

The patch tests were performed in the following manner: a piece of adhesive tape 5.08 x 20 cm. was used. Small pieces of cellophane, about 1.5 cm. square, were placed lengthwise along the strip of tape. On top of these cellophane squares were placed pieces of gauze which were about 1 mm. smaller than the cellophane. These pieces were wetted with the extracts, and the strips of tape were placed on the upper part of the arm between the shoulder and the elbow. At the end of seventy-two hours the tapes were removed and the tests read.

One of the celery workers failed to give reactions to any of the patch tests. He was believed to have reached a stage of hyposensitization. The others, however, displayed reactions varying from redness to "angry" patches with tiny vesicles. Because the celery oil gave a positive test in 67% of the tests, Dr. Rackemann concluded that the oil was definitely the causative agent of the dermatitis.

The pure oil that has been isolated from the celery plant is being used in attempts to reduce the incidence of dermatitis in farm workers who showed positive reactions to the test. Tests are now under way but the results of this attempt at hyposensitization will not be completed until another growing season has passed.

SUMMARY

1. A proximate analysis of Pascal green summer celery was carried out and mannitol was isolated.

2. Various extracts of the plant, together with celery oil itself, were used in patch tests. The volatile oil appears to be the causative factor of dermatitis.

3. The use of various dilutions of the volatile oil in hyposensitization experiments is now in progress.

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Interim Revision Announcements

PHARMACOPEIA OF THE UNITED STATES, FOURTEENTH REVISION

Several U. S. P. XIV interim revisions that have been approved, and their effective dates, are announced in this bulletin.

All interim revision announcements that have appeared since publication of the Third U. S. P. XIV Supplement will be included in the Fourth U. S. P. XIV Supplement, which is scheduled to appear in 1953, and which will be mailed to all registered holders of U. S. P. XIV.

PRECIPITATED CALCIUM CARBONATE

Precipitated Calcium Carbonate, page 98—Change the rubric to read as follows:

Precipitated Calcium Carbonate, dried at 200° for 4 hours, contains not less than 98 per cent of $CaCO_3$.

Change the section, **Loss on drying,** to read:

Loss on drying—Dry Precipitated Calcium Carbonate at 200° for 4 hours: it loses not more than 2 per cent of its weight. See page 733.

Change the first sentence of the section, **Assay,** to read:

Weigh accurately about 1 Gm. of Precipitated Calcium Carbonate, previously dried at 200° for 4 hours, and transfer it to a 250-cc. beaker.

Official and Released December 1, 1952

EPINEPHRINE SOLUTION

Epinephrine Solution, page 214—Make the following changes in the section, **Assay.**

Standard solution of epinephrine—Change this to read:

Standard solution of epinephrine—Weigh accurately 91 mg. of U. S. P. Epinephrine Bitartrate Reference Standard (equivalent to 50 mg. of $C_9H_{13}NO_3$, epinephrine base), previously dried in a vacuum desiccator over sulfuric acid for 18 hours, and transfer it to a 50-cc. volumetric flask with the aid of a solution of 200 mg. of sodium bisulfite in 10 cc. of water. Add water to make exactly 50 cc., and mix well. Each cc. of this solution contains 1.0 mg. of $C_9H_{13}NO_3$ (epinephrine base). Preserve this solution in a tightly-stoppered, hard-glass container, and store under refrigeration and protected from light. Do not use this solution if it shows any evidence of deterioration, such as change in color, or if it has been prepared longer than 6 months.

Test of the animal preparation—Delete entire paragraph.

Comparison of the test dilutions—Change this to read:

Comparison of the test dilutions—Select a dose of the test dilution of the *Standard solution of epinephrine* which upon injection causes a rise in blood pressure between 30 and 50 mm. of mercury. Inject the selected dose at regular intervals of time, which shall be not less than 5 minutes and shall be long enough to allow the blood pressure to return to approximately its former level. When approximately uniform responses have been obtained from two or more injections of the same dose of the Standard, begin alternating injections of the test dilution of the preparation to be assayed, keeping constant the time interval between injections. Vary, in random order, the quantity of the preparation to be assayed so that two of the responses will be expected to be approximately one-fifth less than, two will be expected to be approximately equal to, and two will be expected to be approximately one-fifth greater than the responses to the intervening doses of the Standard. Measure and record to the nearest mm. of mercury the responses observed.

Calculation of the potency—Change the fourth paragraph to read:

If the standard error exceeds 8 per cent of the potency, repeat the assay or increase the number of responses to the Standard and the preparation under assay until the standard error of the combined results is less than 8 per cent of the potency. See *Biological Assays*, page 689.

Official and Released December 1, 1952

PAPAVERINE HYDROCHLORIDE INJECTION

Papaverine Hydrochloride Injection, page 419—Change the section, **pH,** to read:

pH—The *p*H of Papaverine Hydrochloride Injection is not below 3.0.

Official and Released January 1, 1953

POSTERIOR PITUITARY

Posterior Pituitary, page 474—Change the footnote given at the bottom of page 474 to read:

* One U. S. P. Posterior Pituitary Unit is the specific activity of that quantity of U. S. P. Posterior Pituitary Reference Standard which is equivalent in potency to one International Unit of posterior pituitary activity.

Official and Released December 1, 1952

POSTERIOR PITUITARY INJECTION

Posterior Pituitary Injection, page 475—Make the following changes in the section, **Assay.**

Standard solution of posterior pituitary—Change this to read:

Standard solution of posterior pituitary—Prepare a standard solution as follows: Place a U. S. P. Posterior Pituitary Reference Standard tablet, accurately weighed, in a 50-cc. graduated cylinder, dissolve it in 5 cc. of 0.25 per cent acetic acid, and add sufficient 0.9 per cent sodium chloride solution so that each cc. of the resulting solution will represent 0.2 U. S. P. Posterior Pituitary Unit.

Comparison of the test dilutions—Change this to read:

Comparison of the test dilutions—Inject the selected dose of the Standard and repeat at regular intervals of 3 to 10 minutes thereafter. Keep the dose of the Standard constant as long as the recorded blood pressure decreases are between 20 and 40 mm. of mercury, but if necessary, due to changes in sensitivity of the animal, alter the dose appropriately to keep the responses within this range. At the midpoint of each time interval between the doses of Standard, inject a dose of the test dilution of the Injection under assay. Vary, in random order, the quantity of the latter so that two of the responses will be expected to be approximately one-fifth less than, two will be expected to be approximately equal to, and two will be expected to be approximately one-fifth greater than the responses to the intervening doses of the Standard. Measure and record to the nearest mm. of mercury the responses observed.

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WATER FOR INJECTION

Water for Injection, page 664—Add the following section:

Chloride—To 100 cc. of Water for Injection add 5 drops of nitric acid and 1 cc. of silver nitrate T. S.: no opalescence is produced. Water for Injection, packaged and sterilized in glass containers of a capacity of 30 cc. or less, conforms to the following test: to 2 cc. of Water for Injection, placed in a 50-cc. Nessler tube, add 47 cc. of distilled water, 5 drops of nitric acid, and 1 cc. of silver nitrate T. S.: no opalescence is discernible within 10 minutes when the solution is viewed downward over a dark surface.

Official and Released December 1, 1952

By authority of the U. S. P. Board of Trustees and of the Executive Committee of Revision.

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Book Notices

Laboratory Guide in Pharmacology for Pharmacy and Dental Students. By HARALD G. O. HOLCK. Burgess Publishing Co., Minneapolis, Minn., 1951. xiv + 129 pp. Illustrated. 21 x 27.5 cm.

This well-known laboratory guide in pharmacology has been revised in order to bring it into conformity with the U. S. P. XIV and the N. F. IX. In addition to these revisions, some of the experiments have been expanded, e. g., Experiment 36, Introduction to Bioassay; in some areas new experiments have been added, such as Dimercaprol as an Antidote for Arsenicals, Tolerance and Cross-Tolerance to Nostal, Inflammatory Edema, and Trimethadione and Pentylenetetrazol Antagonism.

Film selections have been revised to meet more fully the needs of the pharmacy and dental student. The use of these films permits coverage of topics frequently omitted from the laboratory phase of pharmacology instruction.

Several well-executed new drawings add materially to the value of the guide.

An Inventory of Social and Economic Research in Health. Health Information Foundation, New York, 1952. 197 pp. 23 x 15.5 cm. Price $1.50.

The purpose of this book is to assist all workers in the field of public health who are concerned primarily with its social and economic aspects. It includes classified data on studies conducted by national organizations, by federal, state, and local agencies, and by social science, medical, and health divisions of colleges and universities.

The book is divided into three chapters, including an introduction. The second chapter is composed of an annotated bibliography of social and economic research in health and is divided into five sections. The third chapter includes a project personnel index, a list of organizations conducting the projects previously mentioned in the book and arranged in alphabetical sequence according to states, and other sources of pertinent information about research in health. The Health Information Foundation was organized and is supported by leaders in the drug and allied industries. In this book much valid information on health services, facilities, and personnel has been presented which will be useful to individuals and agencies concerned with health matters from the community to the national level.

Austria-Codex 1952. Edited by DR. OTTO ZEKERT. Österreichischer Apotheker-Verlag, G.m.b.H., Wien, 1952. xlviii + 592 pp. 15 x 10.5 cm.

The 1952 Austria-Codex is a register of specialty preparations distributed under trade mark names in Austria. It includes information concerning each item listed comparable to that found in the *Modern Drug Encyclopedia and Therapeutic Index*, except that the material in the *Codex* is in a highly abbreviated form. *The Austria-Codex* is useful in locating information relative to foreign proprietary preparations not distributed in the United States. Sufficient information is given concerning each preparation so that it is usually possible to determine whether or not a counterpart of it is available in this country. For a more complete review of an

earlier edition of the *Austria-Codex* see THIS JOURNAL, **40**, 302(1951).

International Health Organizations and Their Work. By NEVILLE M. GOODMAN. The Blakiston Company, Philadelphia, 1952. Illustrated. xi + 327 pp. 16 x 23.5 cm. Price $6.50.

This book is intended as a factual survey of health problems which are international in scope. It begins with an account of early efforts in the field of international health and traces the developments in this area through the Health Organization of the League of Nations, the World Health Organization, and other related agencies. It serves adequately to furnish the student or worker in the field of international health with information not heretofore readily available except through documents of various types, many of which are not generally accessible. The book should also serve adequately as a source of information for anyone who wants to become familiar with the history, developments, framework, and aims and accomplishments of the World Health Organization and other agencies concerned with health on an international scale.

Lange's Handbook of Chemistry. 8th ed. Edited by N. A. LANGE and G. M. FORKER. Handbook Publishers, Inc., Sandusky, Ohio. xvi + 1998 pp. 14 x 20.5 cm. Price $7.00.

Since the appearance of the first edition of this comprehensive handbook in 1934, it has undergone seven revisions. As in earlier editions, material previously presented has been thoroughly revised and many changes have been made in numerical values throughout the entire book. Several tables and some subject material not contained in the seventh edition have been added, including ionization potentials of elements, classification of clay minerals, glossary of inorganic chemical nomenclature, titrimetric indicators, molar equivalents of a liter of gas at various temperatures and pressures, and density of moist air. While the principal value of the book is for the professional chemist, it may be recommended as a useful reference book for pharmacists because of the large number of subjects covered and the information presented which is not readily available in handbooks and reference books restricted more or less to the field of pharmacy.

Health Resources in the United States. By GEORGE W. BACHMAN and ASSOCIATES. The Brookings Institution, Washington, D. C.; printed by George Banta Publishing Co., Menasha, Wis., 1952. xvi + 344 pp. 15.5 x 23.5 cm. Illustrated. Price $5.00.

This publication of the Brookings Institution presents a factual account of the personnel, facilities, and services available to serve the nation's health. It will serve as a foundation for future Brookings studies of a number of scientific and social problems relating to health.

In Part I the state of the nation's health during the first half of the present century is reviewed. In Part II data on health personnel are presented, covering not only the professions of medicine, and dentistry, but auxiliary health services including pharmacy. Part III is devoted to an inventory of the structure that has been developed to achieve health objectives. In this section the resources available to meet the problems of mental illness, cancer, heart disease, and other chronic illnesses and programs for special beneficiary classes such as military personnel, veterans, and Indians are discussed in detail. The chapter on health services in industry relating to the health and safety of industrial workers is based upon one of the most extensive surveys ever made in this field.

All of the information is presented objectively. It is emphasized that the study is not intended to furnish a solution to health problems, but to assemble existing information on these problems in order to provide a basic research tool for an appraisal of health services available in the United States.

Occupational Licensing Legislation in the States. Published by the Council of State Governments, 1313 East 60th St., Chicago 37, Ill., June, 1952. 112 pp. Price $3.00.

This report, prepared by the Council of State Governments at the request of the Governors' Conference, makes available in compact form much information on a state-governmental activity that has far-reaching effects.

It presents state-by-state data on occupations licensed, legal qualifications for admission to practice in professions and trades, types of agencies that administer occupational licensing laws, and arrangements for reciprocal, interstate licensing. It deals with the role of professional and trade organizations in the licensing and regulation of their own occupations. Major attention is given to the organization, powers, and functions of state occupational licensing boards and the extent to which the processes of examination and licensure are subject to control by state officials other than members of these agencies. As background for contemporary practices, the report includes a brief history of occupational organization and regulation in past centuries.

The study relates the entire subject of licensure to major public issues that affect all citizens. It is concerned especially with the bearing of licensure on public health and welfare and on the free choice of individuals in selecting their vocations. The area of public policy with which the book deals is one in which there has been an increasing amount of state legislation in recent years. Even as applied to a restricted definition of "occupation", there are now more than 1,200 state occupational licensing laws. Many statutes prescribe educational and experience requirements that must be met by those who desire to practice the occupations in question; many provide for examinations; and the laws commonly include provisions governing practice.

The report just published presents basic data not previously compiled and should be of value to all who are connected with occupational licensing policy.

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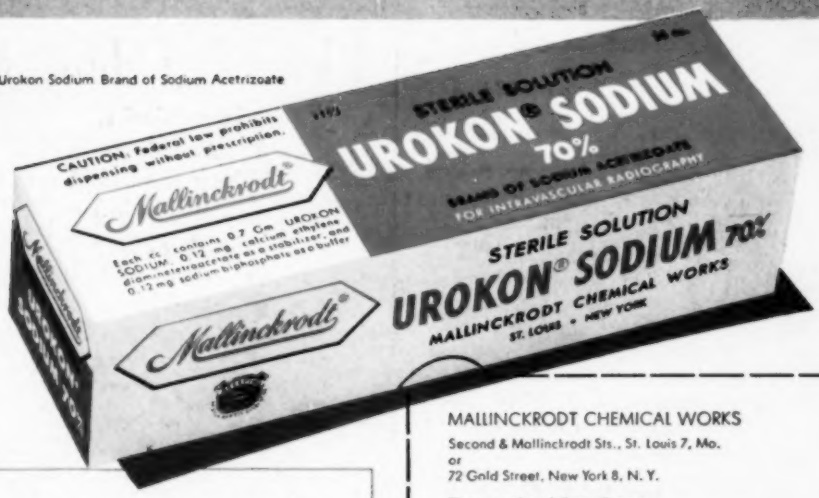
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